CHARACTERIZATION OF DENGUE-2 VIRUS BINDING TO SURFACES OF MAMMALIAN AND INSECT CELLS

BUTSAYA K. THAISOMBOONSUK, EDWARD T. CLAYSON, SOMSAK PANTUWATANA, DAVID W. VAUGHN, AND TIMOTHY P. ENDY

Department of Virology, United States Army Medical Component, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand; Department of Microbiology, Mahidol University, Bangkok, Thailand

Abstract. The binding of dengue type 2 (DEN-2) virus to mammalian (LLC-MK, and Vero) and mosquito (C6/36 and AP61) cell surfaces was investigated by a virus-binding assay using purified 3H-labeled DEN-2 virus. The DEN-2 virus binding to all four cell types was specific and saturable, indicating the presence of a single class of receptors (ranging from 3.7 × 10^4 to 3.5 × 10^4 receptors/cell) with a high affinity for DEN-2 virus (K_d ranging from 98 to 171 pM). Treatment of cell surfaces with certain glycosidases significantly reduced virus binding to mammalian cell lines, but not to the insect cell lines examined. Furthermore, heparin was found to compete with mammalian cell receptors for binding to DEN-2 virus and to inhibit viral infection of mammalian cells, but heparin had no effect on viral binding to or infection of insect cells. These results confirm previous reports suggesting that DEN-2 virus receptors on mammalian cell lines are different from those on insect cell lines.

INTRODUCTION

Dengue emerged as a global health problem in the latter half of the 20th century and is one of the most important arboviral diseases of the tropical and subtropical areas of the world. Dengue (DEN) virus is the causative agent of dengue fever (DF) and dengue hemorrhagic fever (DHF). Four distinct serotypes (DEN-1, DEN-2, DEN-3, and DEN-4) exist; however, they are closely related antigenically by antibody cross-reactivity to other flaviviruses such as Japanese encephalitis virus, yellow fever virus, and tick-borne encephalitis virus. All four dengue serotypes infect humans as the primary vertebrate host and infect Aedes mosquitoes (primarily Aedes aegypti) of the subgenus Stegomyia as the primary mosquito vectors. Dengue virus is characterized as a small (50 nm), enveloped virus containing a single positive strand of RNA and is a member of the family Flaviviridae, genus Flavivirus. The DEN virus genome contains 11,000 nucleotides, lacks a poly(A) tail, and expresses three structural proteins: envelope glycoprotein (E), core (C), and membrane (M). The viral envelope glycoprotein has a molecular mass of 54–60 kD and is thought to be responsible for the initial viral attachment to cells and for mediating cellular entry of the virus. The E protein is the site for several important functions including hemagglutination, infectivity, antibody neutralization, and enhancement for Fc receptor-bearing cells.

The uptake of DEN virus into cells is thought to occur by any of three mechanisms depending on cell type. In cells with Fc receptors (monocytes, macrophages) and in the presence of sub-neutralizing concentration of antibodies, DEN virus uptake is by antibody-dependent Fc receptor-mediated endocytosis. According to this model, DEN virus binds to antibodies that then bind to Fc receptors on cell surfaces. Uptake of virions then occurs by endocytosis. In a variety of mammalian cell lines lacking Fc receptors, DEN virus uptake is reported to occur by receptor-mediated endocytosis. In mosquito cells (C6/36), DEN virus binds to specific cell surface receptors and uptake is reported to occur by fusion of the viral envelope with the cellular membrane. In both of the last two models, DEN virus binds specifically to a cell surface receptor. The nature of the cell surface receptor is not completely understood.

Several putative receptors for DEN-2 virus in various cell lines have been described. Dengue 2 virus appears to bind to a 100-kD protein on the surfaces of human lymphoblastic (K-562) cells, a 65-kD protein on the surfaces of neuroblastoma (N1E-115, SK-N-SH) cells, two proteins of 67 and 80 kD on the surfaces of Aedes albopictus (C6/36) cells, and four proteins of 27, 45, 67, and 87 kD on the surfaces of human macrophages.

Dengue 2 virus recombinant E protein is reported to bind specifically to heparin sulfate on the surfaces of Vero, Chinese hamster ovary (CHO), and baby hamster kidney (BHK) cells. Heparan sulfate is a proteoglycan that is widely distributed in a variety of different cell lines and has binding activity to many microbial agents including bacteria, parasites, and viruses.

Whether the cell surface receptor plays a role in determining tissue tropism is unknown. The Fc receptors are believed to play a role in the severity of disease caused by infection. Whether the specific cell surface receptor plays a role in determining the severity of disease is unknown. Further characterization of the DEN virus receptor on the surfaces of mammalian and insect cells may provide information regarding the diversity of the DEN virus receptor, may lead to a better understanding of the pathogenesis of DF and DHF, and may provide information for the development of antiviral agents or vaccines. The purpose of this study is to characterize the nature, number, binding kinetics, and cell surface receptors for DEN-2 virus in both mammalian (Vero, LLC-MK2) and mosquito (C6/36, AP61) cell monolayers.

MATERIALS AND METHODS

Cell and virus propagation. Vero C1008, LLC-MK2, and C6/36 cell lines were originally obtained from the American Tissue Culture Collection (Manassas, VA). The AP61 cell line (Ae. pseudoscutellaris) was a gift from Dr. Colin Leake (London School of Hygiene and Tropical Medicine, London, United Kingdom) in 1982. Vero and C6/36 cells were grown in Eagles’ minimum essential medium (EMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with 10% heat inactivated fetal bovine serum (HIFBS), and 1%
non-essential amino acid (NEAA). The LLC-MK₂ cells were grown in medium-199 (Gibco-BRL) supplemented with 20% HIFBS. The AP61 cells were grown in MM/V12 media supplemented with 10% HIFBS. Penicillin (100 units/mL) and streptomycin (100 μg/mL) were added to all media. Cells were incubated at either 35°C (LLC-MK₂ and Vero) or 28°C (C6/36 and AP61).

Dengue type 2 virus (New Guinea C strain) was propagated in ICR suckling mouse brain for 30–35 passages before passage into C6/36 cells for an additional 8–10 passages.²²

**Preparation of purified dengue virus.** Confluent C6/36 monolayers in T162 flasks (Costar; Corning, Corning, NY) were inoculated with DEN-2 virus at a multiplicity of infection of 0.1 for 45 minutes at room temperature on a rocker platform. Maintenance medium (5% HIFBS, EMEM, 1% NEAA) was then added and the monolayers were incubated at 32°C in a Forma Scientific (Marietta, OH) incubator without CO₂. When preparing radiolabeled DEN-2 virus, the maintenance media was 5% HIFBS-EMEM without L-leucine supplemented with 1% NEAA, 20% normal L-leucine (Gibco-BRL), and 1-[3,4,5-³H(N)] leucine (4.1 mCi/mL) (Sigma, St. Louis, MO). Tissue culture supernatant fluids were collected at the day of highest cytopathic effect (typically five days post-inoculation) and clarified at 1,000 × g on a Sorvall (Asheville, NC) (RC-3B plus) centrifuge at 4°C for 10 minutes. Virus particles were precipitated from supernatants using polyethylene glycol (PEG, molecular weight = 8,000; Sigma) using 7% PEG and 2.4% NaCl (w/v at the final concentration), while stirring on ice for 2 hours, followed by centrifugation at 16,800 × g on a Sorvall (RC-5B using a GSA rotor) centrifuge at 4°C for 45 minutes. The virus pellet was resuspended in 1:100 TNE (10 mM Tris-HCl, 100 mM NaCl, 1mM EDTA, pH 7.8). The DEN-2 virus was further purified by overlaying a 1:30 virus suspension onto a 15–60% stepwise sucrose gradient (initial 1 mL of 15% followed by 2 mL each of 30–60% in 5% increments [w/w] in phosphate-buffered saline [PBS] without Ca/Mg; BioWhittaker, Walkersville, MD) and ultracentrifugation at 52,000 × g (Beckman model L8-M, SW 27 rotor) at 4°C for 18 hours. Fractions in 1-mL aliquots were collected from the bottom of the gradient. Fractions were tested by viral plaque assays in LLC-MK₂ cells,²³ hemaggulination assay, and by liquid scintillation spectrometry to determine the fractions with peak virus concentration. These fractions were pooled and retested for viral infectivity (3.0 × 10⁷ plaque-forming units [PFU]/mL), hemaggulination activity (≥ 2,560) and specific activity (2,156 cpm/mL). In addition, total viral RNA was extracted and resuspended as described.²⁴ The optical density (OD) of the suspension was measured at an OD260 and OD280. The concentration of DEN-2 virus particles in the pooled fraction (7.0 × 10¹¹ virus particles/mL) was calculated based on the reported molecular weight of DEN-2 viral RNA (3.3 × 10⁵).²⁵

**Binding assay.** Cell monolayers were prepared in 96-well plates (Costar 3596; Corning) in growth medium supplemented with 25 mM HEPES. The cells were incubated in 5% CO₂ incubators at 35°C (LLC-MK₂ and Vero) or 28°C (C6/36 and AP61). Confluent cell monolayers were washed once and replaced with cold bovine serum albumin (BSA)–EMEM (0.8% BSA in EMEM, 25 mM HEPES, pH 6.0) for 1 hour at 4°C. Monolayers were then exposed to purified ³H-labeled DEN-2 virus at indicated concentrations and incubated on ice for 2 hours on a rocker platform. The inoculum was removed and cell monolayers were washed three times with cold BSA-EMEM. The monolayers were solubilized with 1% sodium dodecyl sulfate (Sigma), transferred to glass fiber filter strips (Bellco, Vineland, NJ), and placed in mini poly Q vials (Beckman, Fullerton, CA) containing 3 mL of Cytoscint (ICN Biomedicals, Irvine, CA). Radioactivity was measured by liquid scintillation spectrometry (Beckman LS 1801). All assays were performed in triplicate.

**Enzyme treatment of cell monolayers.** Various phospholipases, proteases, and glycosidases were diluted in EMEM-25 mM HEPES at the recommended pH. Confluent cell monolayers in 96-well plates were treated with the enzyme dilutions for 15 minutes as described.²⁶ Enzyme solutions were removed, monolayers were washed, and the plates were observed by light microscopy for monolayer integrity. The binding assay was performed and cell-associated radioactivity was determined as described earlier in this report. The results from 4–5 10-fold serial enzyme dilutions were analyzed using GraphPad PRISM™ software version 2.0 (GraphPad Software, Inc., San Diego, CA) to determine the concentration of enzyme that inhibits 50% of virus binding to cell surfaces (EC₅₀). If enzyme treatment did not reduce virus binding, the highest concentration of enzyme examined is indicated. In a few instances, a high concentration of enzyme resulted in partial or complete detachment of cells from the plate. In these circumstances, results were not used in the analysis. All enzymes were obtained from Boehringer Mannheim Biochemicals (Mannheim, Germany), except for heparinase I, which was obtained from Sigma and trypsin (1:250), which was obtained from Gibco-BRL.

**Heparin-blocking assays.** The ability of heparin to block binding and/or penetration of DEN-2 virus to cells was examined using a binding assay, a plaque assay, and by indirect immunofluorescence. In one set of experiments, confluent cell monolayers in 96-well plates were washed once and incubated with heparin-virus mixtures (containing the indicated concentrations of heparin [Sigma] diluted in BSA-EMEM and a constant amount of purified ³H-labeled DEN-2 virus) at 4°C for 2 hours on a rocker platform. The inoculum was removed and cell-associated radioactivity was determined using the binding assay as described earlier in this report.

In a separate set of experiments, confluent LLC-MK₂ or Vero cell monolayers in 24-well plates (Costar) were incubated with heparin-virus mixtures on a rocker platform for 90 minutes. The heparin-virus inocula were removed and a first overlay media (1.8% of low-melting point agarose [Gibco-BRL] equally mixed with 10% HIFBS in 2× modified Eagle’s medium adjusted pH to 8.2) were added. The plates were incubated at 35°C in an atmosphere of 5% CO₂ for 3–5 days. A second overlay medium (without HIFBS/NaHCO₃) including 0.01% neutral red (Sigma) was added as staining for plaque counting. Plaques were counted over a standard light box. All values are triplicates of three runs. The AP61 and C6/36 cells were not used in this set of experiments because these cells do not produce visible plaques.

For indirect immunofluorescence staining, confluent cell monolayers in eight-well Permanox® chamber slides (Nunc Laboratory-Tek, Napierville, IL) were washed once with BSA-EMEM, pH 7.3, and incubated with heparin-virus mixtures for 2 hours at room temperature on a rocker platform. The heparin-virus inocula were removed and the monolayers were washed once. Maintenance medium was added and the
cell monolayers were incubated at 35°C in a 5% CO₂ incubator for 24 hours. Cell monolayers were washed twice with Hank’s balanced salt solution, pH 7.4, and twice with PBS, pH 7.5. Cell monolayers (on slides) were air-dried and fixed with cold acetone for 10 minutes. The slides were washed with 1% BSA in PBS, pH 7.5, and incubated with mouse IgG antibody to DEN-2 virus (diluted 1:20 from a pool of ascitic fluids collected from several mice as described previously) at 35°C in a humid box for 30 minutes. The slides were washed in PBS, pH 7.5, with 0.05% Tween 20 (PBST) for 10 minutes and incubated with fluorescein-conjugated sheep anti-mouse IgG (diluted 1:40; Sigma) at 35°C in a humid box for 30 minutes. The slides were washed in PBST for 10 minutes and fixed with 0.1% 3-phenylendiamine in 90% glycerol-PBS, pH 8.0. The slides were observed with an Olympus (Melville, NY) Universal Research BX51:BX2 series microscope and photographed with an automated photomicrographic system (Model PM-30; Olympus).

Statistical analysis. Statistical analysis was performed using SPSS for Windows, version 8.0 (SPSS Inc., Chicago, IL) and Statistica, version 5.0 (StatSoft Inc., Tulsa, OK). The concentration of enzyme that inhibits 50% of virus binding to cell surfaces (EC50) was determined using GraphPad PRISM™ software (version 2.0). The effect of increasing concentrations of heparin and infectivity as measured by viral plaques per well was analyzed by linear regression.

RESULTS

Determination of the optimal pH for DEN-2 virus binding to cell surfaces. The effect of pH on the binding of DEN-2 virus to mammalian or mosquito cell surfaces (as represented by LLC-MK2 and C6/36 cell lines, respectively) was determined for a series of pH values between pH 5.0 and 9.5 (Figure 1). The optimum pH range for DEN-2 virus binding to both LLC-MK2 and C6/36 cell surfaces was between 5.5 and 6.0. Virus binding to both cell lines decreased sharply at pH values greater than 6.0. Subsequent virus binding experiments for both mammalian and mosquito cell lines were conducted at pH 6.0, with a few exceptions as noted.

Saturation of DEN-2 virus binding sites on the surfaces of mammalian and insect cells. To determine if a finite number of receptor sites are present on the surfaces of mammalian (LLC-MK2, Vero) and insect (C6/36, AP61) cell lines, confluent cell monolayers were exposed to increasing amounts of purified unlabeled DEN-2 virus before being exposed to a constant amount of purified 3H-labeled DEN-2 virus. If specific receptors are responsible for DEN-2 virus binding to cell surfaces as previously observed by others, then the receptor should exist in a finite number on the cell surface and excess unlabeled virus should be able to compete with labeled virus for the receptor. Unlabeled DEN-2 virus at concentrations of 1 × 10⁴ PFU/cell and higher was able to effectively compete with 3H-labeled DEN-2 virus on all four cell lines examined, indicating that a finite number of binding sites are present on cell surfaces.

To demonstrate the saturability of a finite number of cell receptors, cell monolayers were exposed to increasing amounts of 3H-labeled DEN-2 virus and used in the binding assay. The calculated number of virions bound per cell were plotted verses the input multiplicity of infection to produce binding curves. Saturation of receptors did occur at high input multiplicities of virus (Figure 2) in all four cell lines examined. The data in Figure 2 were replotted by the method of Scatchard28 to estimate the average number of receptors per cell and the affinity of the receptor for DEN-2 virus. The x intercept in a Scatchard plot indicates the number of receptors per cell. The number of receptors per cell ranged from 0.96 to 1.05 (Figure 4), indicating no cooperativity between receptors in any of the cell lines examined. The calculated Hill coefficients were very similar and ranged from 0.96 to 1.05 (Figure 4), indicating no cooperativity between receptors in any of the cell lines examined.

Inhibition of DEN-2 virus binding by treatment of cell surfaces with various enzymes. To characterize the biochemical nature of the cell receptor for DEN-2 virus, mammalian and insect cell monolayers were treated with a variety of enzymes to remove cell surface molecules. The receptor activity remaining on the cell monolayers after enzyme treatment was measured with the binding assay using 3H-labeled virus. Treatment of any of the cell surfaces with phospholipase A₂ (EC₅₀ = 100 μ/mL), phospholipase C (EC₅₀ = 1 U/mL), or phospholipase D (EC₅₀ = 2,000 μ/mL) had little or no effect on virus binding.

Among the proteases tested, chymotrypsin A₂ (EC₅₀ = 20–53 μ/mL), trypsin (EC₅₀ = 41–113 μ/mL), pronase
EC50/H11229 - 10/H9262/mL), subtilisin (EC50/H11229 - 10/H9262/mL), bromelain (EC50/H11229 - 10/H9262/mL), papain (EC50/H11229 - 10/H9262/mL), endoproteinase Glu-C (EC50/H11229 - 10/H9262/mL), and endoproteinase Lys-C (EC50/H11229 - 5 U/mL) significantly reduced virus binding to the surfaces of all four cell lines examined. No statistical differences in EC50 values were observed between mammalian and insect cell lines. Treatment of cell surfaces with pepsin (1,000 U/mL) or endoproteinase Arg-C (40 U/mL) had no effect on virus binding to the surfaces of any of the cell lines examined.

Among the glycosidases tested, amyloglucosidase (≤ 50 U/mL), β-galactosidase (≤ 150 U/mL), N-acetyl-β-d-glucosaminidase (≤ 5 U/mL), endo-β-galactosidase (≤ 100 mU/mL), N-glycosidase F (≤ 20 U/mL), O-glycosidase (≤ 40 U/mL), and cereamide glycanase (≤ 10 mU/mL) had no effect on virus binding to the surfaces of any of the cell lines examined. However, β-glucosidase (EC50 = 5–13 U/mL), neuraminidase (EC50 = 5–6 U/mL), and heparinase I (EC50 = 23–49 U/mL) reduced DEN-2 virus binding to the surfaces of LLC-MK2 and Vero cells, but not to the surfaces of C6/36 and AP61 cells.

Blocking of DEN-2 virus binding to mammalian cells, but not insect cells, with heparin. The ability of heparinase I to reduce DEN-2 virus binding to mammalian cells suggests that DEN-2 virus may bind to heparin sulfate moities on the cell surface. If so, heparin should compete with the receptor for binding to DEN-2 virions. The ability of heparin to compete with the receptor for binding to DEN-2 virus was examined in all four cell lines by exposing confluent cell monolayers with heparin-virus mixtures containing increasing amounts of heparin and a constant amount of 3H-labeled DEN-2 virus. The ability of virus to bind to cell surfaces was measured with the binding assay. Heparin blocked virus binding to both mammalian cell lines in a dose dependent manner (IC50 = 5 μ/mL). However, heparin (≤ 1,000 μ/mL) had no effect on virus binding to either insect cell lines (Figure 5).

To determine whether heparin blocks DEN-2 virus infection in cells, LLC-MK2 and Vero cell monolayers were exposed to heparin-virus mixtures. Viral infectivity was determined 3–5 days later by a plaque assay. Heparin blocked viral plaque formation in both mammalian cell lines in a dose-dependent manner similar to that seen for the binding assay (IC50 = 3–10 μ/mL). This experiment was not conducted using the two insect cell lines (C6/36, AP61) because viral plaques are not visible on these cell lines.

To determine whether heparin could effectively block infection in insect cells, confluent monolayers (all four cell lines) were exposed to heparin-virus mixtures. After incubation for 24 hours, the synthesis of viral proteins was examined by indirect immunofluorescence. Heparin significantly re-
duced the number of cells expressing viral proteins in both mammalian cell lines, but had little to no effect on either insect cell line (Figure 6).

**DISCUSSION**

The study of the early interactions of viruses with cell surfaces may lead to a greater understanding of viral tropism and pathogenesis. We used a virus binding assay and Scatchard analysis to gain information on the kinetics of the early DEN virus-cell interactions on both mammalian and insect cell cultures. Similar methods have successfully been used to study the binding of Rauscher murine leukemia virus envelope glycoprotein to KA31 cell membrane fractions, the binding of Semliki Forest virus particles to BHK cell monolayers, the binding of vesicular stomatitis virus to Vero cell monolayers, the binding of reovirus particles to several cell monolayer types, the binding of adenovirus particles to HeLa cell monolayers, the binding of Moloney murine leukemia virus envelope glycoprotein to murine L-cell membrane fractions, the binding of hepatitis B virus envelope glycoprotein (small S protein) to 30 different cell monolayers, and the binding of simian virus 40 to several monkey kidney cell types. Likewise, we used enzyme treatment of cell membrane surfaces and the virus binding assay to gain information on the biochemical nature of the cellular receptor on both mammalian and insect cell cultures. Our studies point out several similarities and differences among mammalian and insect cell receptors.

We found DEN-2 virus binding to cell surfaces of both mammalian and insect cells to be specific and saturable, indicating that a finite number of receptors exist on the surfaces of all four cell lines examined. Scatchard analysis of the binding of DEN-2 virus to each of the four cell line surfaces showed that the number of binding sites per cell ranged from $4.3 \times 10^3$ to $6.6 \times 10^3$ in the two insect cell lines and from $1.4 \times 10^4$ to $3.5 \times 10^4$ in the two mammalian cell lines. These values are within the range reported for other viral cell surface receptors. Scatchard analysis also showed that the cell surface receptors have a high affinity for DEN-2 virus. Dissociation constants ($K_d$) ranged from 98 pM to 125 pM in the two insect cell lines and from 142 pM to 171 pM in the two mammalian cell lines. The $K_d$ values are also within the range (4–2,900 pM) reported for other viral cell surface receptors.

Hill analysis of the binding of DEN-2 virus to each of the four cell lines yielded coefficients ranging from 1.00 to 1.05 in the two insect cell lines and from 0.96 to 0.98 in the two mammalian cell lines. The Hill coefficients in this range indicate no cooperativity between receptors in any of the cell lines examined. The apparent differences in the number of cell surface receptors, the $K_d$ values, and the Hill coefficients between insect and mammalian cell lines are small and may not be statistically significant.

We found DEN-2 virus binding to cell surfaces of both mammalian and insect cells to be unaffected by treatment of the cell surfaces with three phospholipases, suggesting that phospholipids do not play an important role in virus-receptor binding to any of the cell types examined. Dengue type 2 virus binding to both mammalian and insect cell lines was affected by treatment of cell surfaces with a variety of proteases, suggesting that protein is an important component of the DEN-2 virus cell receptor in all cell lines examined. We observed apparent differences in the effects of two proteases (bromelain, papain) on the binding of DEN-2 virus to mammalian...
verses insect cell lines; however, the differences in EC<sub>50</sub> values were not statistically significant. Further characterization of protein structure of both cell types using various enzyme families/activities is in progress.

We detected differences in the ability of certain glycosidases to affect DEN-2 virus binding to mammalian verses insect cell lines. While treatment of cell lines with several glycosidases had no effect on virus binding to the surfaces of any of the cell lines examined, β-glucosidase, neuraminidase, and heparinase I reduced DEN-2 virus binding to the surfaces of both mammalian cell lines, but not to the surfaces of either insect cell line examined. These results suggest that certain carbohydrates play an important role in DEN-2 virus binding to the surfaces of mammalian cells, but not to the surfaces of insect cells. Some insight into the carbohydrate moieties on mammalian cells necessary for DEN-2 virus binding may be gained by looking at the specificities of the three glycosidases that reduced DEN-2 virus binding. The enzyme β-glucosidase, cleaves β-linked terminal hexoses (glucose, galactose, fucose), neuraminidase cleaves terminal sialic acid, whereas heparinase I cleaves heparin sulfate residues from carbohydrate backbones. This suggests that the carbohydrate moiety on mammalian cells necessary for DEN-2 virus binding contains β-linked terminal glucose, galactose, or fucose; terminal sialic acid residues; and heparin sulfate residues.

The inability of neuraminidase to affect DEN-2 virus binding to insect cells is consistent with previous observations. Salas-Benito and del Angel<sup>37</sup> reported that treatment of cell surfaces with neuraminidase did not reduce DEN-4 virus binding to C6/36 cells. Sialic acid is not found in mosquito cells, and the cells lack the enzyme sialyl transferase, which

**FIGURE 4.** Hill analysis of dengue type 2 virus binding to mammalian and insect cell surfaces. The data in Figure 2 were replotted by the method of Hill<sup>29</sup>. B is the number of bound virions per cell, n is the number of receptors per cell as determined in Figure 3, and F is the number of virions remaining unbound per well. The Hill coefficient was calculated from the slope of the line. A value of 1.00 is indicative of no cooperation between receptors. A, LLC-MK<sub>2</sub> cell line, B, Vero cell line, C, C6/36 cell line, D, AP61 cell line.

**FIGURE 5.** Effect of heparin on blocking of dengue type 2 (DEN-2) virus binding to cell surfaces. Triplet cell monolayers in 96-well plates were incubated with heparin-virus mixtures (containing the indicated concentrations of heparin and saturating amounts of <sup>3</sup>H-labeled DEN-2) for 2 hours at 4°C. After the inoculum was removed, cell-associated radioactivity was determined by liquid scintillation spectrometry. Results are shown as percentage of control (no heparin) virus binding compared with heparin concentration in the heparin-virus mixture.
transfers sialic acid residues to glycoproteins. Likewise, our observations that treatment of cell surfaces with heparinase I reduces DEN-2 virus binding to mammalian cells and that heparin competes with mammalian cell surface receptors are consistent with previous observations. Recombinant E protein is reported to bind specifically to heparin sulfate on the surfaces of Vero, CHO and BHK cells. Therefore, it is not surprising that heparinase I would reduce DEN-2 virus binding to these cell surfaces nor is it surprising that heparin would compete with the cell receptor for binding to DEN-2 virus. In addition, although heparin sulfate is a major component of mammalian cell surfaces, it is not believed to be found on insect cells.

Successful transmission of DEN virus in the wild involves alternate virus replication in mosquitoes and mammals. The virus must be able to recognize cell surface receptors in both cell types. Our results suggest that the cell surface receptors used by DEN virus mammalian cells are different than those used in insect cells. The mammalian cell surface receptor contains sialic acid and heparin sulfate, whereas the insect cell receptor does not. Therefore the DEN virus envelope protein may have two receptor binding domains, one that recognizes heparin sulfate/sialic acid moieties on mammalian cells and another that recognizes an unidentified moiety in insect cells. Further work is needed to further characterize the mammalian cell receptor and to identify the insect cell receptor for DEN virus.

Received August 29, 2003. Accepted for publication September 25, 2003.

Acknowledgments: We thank Dr. Ananda Nisalak and Dr. Khin Saw Aye Myint for their valuable suggestions, Somsak Imlap for cell culture assistance, and Dr. Mammen P. Mammen for his review of the manuscript.
Financial support: This work was supported by the United States Army Medical Research and Materiel Command, (Fort Detrick, Frederick, MD).

Disclaimer: The opinions or assertions contained herein are the private views of the authors and are not to be construed as reflecting the official views of the United States Army or the Department of Defense.

Authors' addresses: Butsaya K. Thaisomboonsuk, Department of Virology, United States Army Medical Component, Armed Forces Research Institute of Medical Sciences, APO AP 96546 (from the United States) or 315/6 Rajvithi Road, Bangkok 10400, Thailand (from outside the United States), Telephone: 66-2-644-6464, Fax: 66-2-644-4760, E-mail: ButsayaT@afrims.org Edward T. Clayson, Project Management Office, Chemical Biological Medical Systems, 64 Thomas Johnson Drive, Frederick, Maryland 21702, Telephone: 301-619-8402, Fax: 301-619-8025, E-mail: Edward.Clayson@det.army.mil. Somsak Pantuwatana, Department of Microbiology, Mahidol University, Bangkok 10400, Thailand, E-mail: pantuwan@bu.ac.th. David W. Vaughn, VA Infectious Diseases Research Program, Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Frederick, MD 21702-5012, E-mail: David.Vaughn@amedd.army.mil. Timothy P. Endy, Walter Reed Army Institute of Research, Rm 3528, 503 Robert Grant Ave., Silver Spring, MD 20910-7500, E-mail: Timothy.Endy@na.amedd.army.mil.

Reprint requests: Butsaya K. Thaisomboonsuk, Department of Virology, United States Army Medical Component, Armed Forces Research Institute of Medical Sciences, APO AP 96546 (from the United States) or 315/6 Rajvithi Road, Bangkok 10400, Thailand (from outside the United States).

REFERENCES


