Correlation of E protein binding with cell susceptibility to dengue 4 virus infection

Robert Anderson,1* Alan D. King2 and Bruce L. Innis2

1Department of Microbiology and Infectious Diseases, University of Calgary, Calgary, Alberta T2N 4N1, Canada and 2Department of Virology, Armed Forces Research Institute of Medical Science, 315/6 Rajvithi Road, Bangkok 10400, Thailand

Supernatant culture fluids from dengue virus type 4 (DEN-4)-infected cultures of monkey kidney Vero cells and Aedes albopictus C6/36 cells contained the virion structural proteins; secreted NS1 was found only in supernatants from infected Vero cells. Using supernatant culture fluids from [35S]methionine-labelled, virus-infected Vero and C6/36 cells, binding of radiolabelled viral proteins was examined with various cell lines varying in susceptibility to DEN-4 infection. Binding of viral E protein was observed with the highly infectible Vero and LLC-MK2 cell lines, whereas a very small degree of binding was seen with four other cell lines (mouse fibroblast L929, bovine kidney MDBK, human hepatoma Hep G2 and primary human endothelial cells) which are less susceptible to DEN-4 infection. The results suggest that cell susceptibility to DEN-4 may be determined largely at the stage of virus binding, i.e. by the presence of a cell receptor capable of binding viral E protein.

The mechanisms underlying cell tropism for flaviviruses remain poorly understood. Most flavivirus infections may be mild or even asymptomatic, but severe clinical manifestations, when they occur, generally include a haemorrhagic or encephalitic component. Such disease severity is of global concern and lends impetus to the identification of viral determinants which determine tissue susceptibility to infection and pathogenicity.

Flavivirus entry into permissive cells apparently occurs by an endocytic mechanism following virus binding (Gollins & Porterfield, 1985, 1986; Kimura et al., 1986), although evidence for an alternative plasma membrane penetration process has also been presented (Hase et al., 1989). The cell-binding event is presumed to involve the viral E glycoprotein, although this has not been directly demonstrated. Consistent with an involvement of the E protein in the process of cell entry is the finding of an intramolecular region (residues 98 to 111), conserved among known flaviviral E proteins, which is possibly involved in low pH-catalysed membrane fusion (Roehrig et al., 1989) as well as evidence for a pH-independent conformational change (Guirakhoo et al., 1989; Kimura & Ohyama, 1988).

We demonstrate here the direct binding of dengue-4 virus (DEN-4) E protein to cells of various derivation and show that the degree of E protein binding correlates with cell susceptibility to the virus. To our knowledge, this study shows the first reported cell-specific interaction with flavivirus polypeptides and provides evidence for the importance of E protein attachment in determining cell susceptibility to DEN virus infection.

Monolayer cultures in 24-well plates of Aedes albopictus C6/36 (Igarashi, 1978), African green monkey kidney Vero (Yasumura & Kawakita, 1963) and human hepatoma Hep G2 (Aden et al., 1979) cells were inoculated with DEN-4 (814669 strain) at an m.o.i. of 3. Medium used for C6/36, Vero and Hep G2 cells was Eagle's MEM supplemented with 2 mM-glutamine and 10% heat-inactivated foetal calf serum (FCS; Gibco). After incubation at either 30 °C (C6/36 cells) or 35 °C (Vero, Hep G2 cells) for 48 h, cultures were radiolabelled with [35S]methionine (50 μCi/ml) for 24 h. Supernatant fluids were then harvested and clarified by low-speed centrifugation (5 min at 1000 g). Aliquots taken for plaque assay showed levels of DEN-4 of $3 \times 10^6$ p.f.u./ml, $5 \times 10^6$ p.f.u./ml and $6 \times 10^3$ p.f.u./ml in the supernatant media taken from Vero, C6/36 and Hep G2 cells, respectively. Aliquots (25 μl) of the supernatants were immunoprecipitated by mixing with 5 μl mouse anti-DEN antiserum in immunoprecipitation (IP) buffer (10 mM-phosphate buffer pH 7.2, 0.15 M-NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS). Following overnight incubation at 4 °C, 100 μl of an 8% suspension of fixed Staphylococcus aureus cells was added, incubated 1 h at 4 °C, microfuged and washed twice with IP buffer. Final pellets were resuspended (no boiling) in sample buffer...
Short communication

1 2 3 4 5 6

Fig. 1. SDS-PAGE fluorograph of immunoprecipitated, radiolabelled proteins secreted into culture fluids of C6/36 (lanes 1 and 4), Vero (lanes 2 and 5) or Hep G2 cells (lanes 3 and 6). Cultures were either mock-infected (lanes 1 to 3) or infected with DEN-4 (lanes 4 to 6) and labelled with [35S]methionine from 48 to 72 h post-infection (p.i.). Culture fluids were clarified and immunoprecipitated with murine anti-DEN antiserum. The positions of the dimeric NS1 and E are marked (observed Mr 82000 and 54000 to 55000, respectively).

As shown in Fig. 1, E protein appeared in the supernatant medium within 2 h of chase. Maximum production of virion-associated E protein occurred by 8 to 12 h of chase. Similar kinetics of the appearance of the E protein into supernatant media from flavivirus-infected cells have been reported for JE virus (Mason, 1989). The DEN-4 E protein was almost exclusively associated with the pelletable (i.e. virion) fraction following ultracentrifugation (Fig. 2). Only a small proportion (<5% as estimated by scintillation counting of the corresponding bands excised from the dried gel) of the total E protein present in the supernatant medium at 12 h of chase was present as a ‘soluble’ form (Fig. 2, lane 5 of supernatant fraction). Greater than 93% of the virion infectivity was present in the pellet fraction, as determined by plaque assay of total supernatant and pellet fractions.

The major aim of this study was to demonstrate binding of DEN-4 proteins (particularly the E protein) to cells and to determine whether such binding correlated with cell susceptibility to infection. Monolayer cultures of Vero, LLC-MK2 (Hull et al., 1962), Hep G2, mouse fibroblast L929 (Sanford et al., 1948), bovine kidney MDBK (Madin & Darby, 1958) and human endothelial cells were established (human endothelial cell cultures were derived from a single umbilical vein and propagated in RPMI-1640 medium supplemented with 2 mM-
Fig. 2. Kinetics of appearance of radiolabelled DEN-4 proteins in culture fluids from infected Vero cells. Cell monolayers, in 24-well plates were inoculated with DEN-4 and pulse-labelled for 1 h with [35S]methionine at 48 h.p.i. and subsequently chased for 1, 2, 4, 8 or 12 h (lanes 1 to 5, respectively). Aliquots of clarified culture fluids (total) were separated by ultracentrifugation (100000 g) into supernatant (super) and pellet fractions. All samples were solubilized in IP buffer and immunoprecipitated using mouse anti-DEN antiserum. Immunoprecipitates were resolved by SDS–PAGE and radiolabelled proteins visualized by fluorography. The positions of (NS1)2, E and C are indicated (observed Mr 82000, 55000 and 12000, respectively). A small amount of monomeric NS1 (observed Mr 41000) is visible below the E protein. The M protein (predicted Mr 8000) which is poorly detectable in flavivirions (see also Boege et al., 1983) could not be unequivocally identified.

glutamine, 20% FCS, endothelial cell growth factor and heparin). To establish relative susceptibility to infection, cultures were inoculated with DEN-4 at an m.o.i. of 3, incubated 48 h at 35 °C and examined for immunofluorescent staining of viral antigen using polyclonal anti-DEN mouse serum (preadsorbed against Vero cell lysate) and fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Under the conditions used, Vero and LLC-MK2 cells were the most susceptible to DEN-4 infection, with more than 85% of cells displaying viral antigen. Hep G2 and human endothelial cells were less infectible by DEN-4 (approx. 10% of cells containing viral antigen), and L929 and MDBK cells were the least susceptible (less than 5% viral antigen-positive cells). From these results, it is clear that the monkey kidney Vero and LLC-MK2 cells are much more permissive to DEN-4 infection than any of the other cell lines examined.

Radiolabelled culture fluids prepared from [35S]methionine-labelled Vero cells (either mock- or DEN-4-infected) were applied to monolayers of MDBK, Vero and L929 cells and allowed to adsorb for 1 h at 4 °C to allow binding of radiolabelled proteins to the cells. Cultures were washed four times with medium (RPMI supplemented with 5% FCS) and the cells were subsequently scraped with a Teflon policeman, microfuged and the cell pellets were resuspended in 100 μl IP buffer. As shown in Fig. 3, DEN-4 E protein bound well to Vero cells but only weakly to either MDBK or L929 cells.

In a study analogous to that described above we used culture fluid supernatants from [35S]methionine-labelled DEN-4-infected C6/36 cells. In addition to Vero, MDBK and L929 cells, we employed Hep G2 and human endothelial cell cultures for assays of viral protein binding. These five cell types were grown to confluence in individual wells of a 24-well plate. Monolayer cultures were overlaid with radiolabelled culture fluid from [35S]methionine-labelled DEN-4-infected C6/36 cells for 1 h at 4 °C, washed and harvested as above. Aliquots of
Fig. 3. Binding of [35S]methionine-labelled, mock- (lanes 2, 4, 6 and 8) or DEN-4-infected (lanes 1, 3, 5 and 7) Vero cell supernatant culture fluids to different cells. Monolayer cultures of MDBC (lanes 3 and 4), Vero (lanes 5 and 6) and L929 (lanes 7 and 8) cells were overlaid with radiolabelled culture fluid for 1 h at 4 °C, subsequently washed and solubilized for analysis by fluorographic SDS-PAGE. Samples were standardized according to equal numbers of cells.

Fig. 4. Binding of [35S]methionine-labelled, DEN-4-infected C6/36 cell culture fluids to five different cell types. Monolayer cultures of MDBC (lanes 1 to 3), Vero (lanes 4 to 6), Hep G2 (lanes 7 to 9), human endothelial cells (lanes 10 to 12) and L929 cells (lanes 13 to 15) were overlaid with radiolabelled culture fluid for 1 h at 4 °C, subsequently washed and solubilized in IP buffer. For each cell line, radiolabelled proteins are shown from the unbound culture fluid (lanes 1, 4, 7, 10 and 13) as well as cell-bound material before (lanes 2, 5, 8, 11 and 14) and after (lanes 3, 6, 9, 12 and 15) immunoprecipitation with mouse anti-DEN antiserum. Samples were standardized according to equal numbers of cells. Lane 16, culture fluid from [35S]methionine-labelled DEN-4-infected Vero cells, showing (NS1)2 and E protein bands.

the cell extracts were taken for immunoprecipitation and SDS-PAGE fluorography. As shown in Fig. 4, the highest degree of viral protein binding occurred with Vero cells. Vero cells bound DEN-4 E protein as shown by comigration with a protein standard and immunoprecipitation with anti-DEN mouse antiserum. An immunoprecipitable protein band of slightly lower Mr (faster migration) was also observed in the Vero cell-bound fraction (Fig. 4). The identity of this protein is uncertain, but it may represent a proteolytically cleaved form of cell-bound E. However this protein did not appear in all experiments and therefore its significance if any is unknown. From the results of Fig. 4, it is clear that the highly permissive Vero cell line binds DEN-4 E protein much more efficiently than any other (less permissive) lines tested. The amount of DEN-4 E protein bound by another line of monkey kidney cells, LLC-MK2, which shows permissiveness to DEN-4 comparable to that of Vero cells, was similar to that bound by Vero cells (data not shown). Since flaviviral E protein produced in mosquito (e.g. C6/36) and mammalian (e.g. Vero) cells differs in the carbohydrate composition of the oligosaccharide chains (e.g. Mason, 1989) it appears from the results of Fig. 3 and 4 that such carbohydrate differences play a minor role in binding of virion-associated E protein to susceptible cells.

Our study provides direct evidence for the importance of the E protein in binding to susceptible cells and suggests that cell tropism of DEN virus is determined, in several cell types, by the presence of an E protein receptor. The observation that the relatively closely related cell lines Vero and LLC-MK2 showed much higher levels of E protein binding than did the other cell lines tested suggests that they possess high levels of the candidate receptor and are a good source for its isolation.
The identification and characterization of this receptor will thus be of fundamental importance to understanding and controlling infection and disease caused by DEN virus.

Although considerable progress has been made in the identification of cell receptors for a number of viruses, little is currently known regarding the nature of flavivirus receptors. Not surprisingly, viruses that infect numerous host organisms and cell types may recognize more than one receptor molecule, as demonstrated for Sindbis virus, a member of the alphavirus genus (Smith & Tignor, 1980; Wang et al., 1991). Moreover, the ability of arthropod-borne viruses, such as the alpha- and flaviviruses to replicate in cells of both vertebrate and invertebrate origin suggests the likelihood of divergent cell receptors participating in virus attachment. Studies are in progress to gain insights into such possible receptor diversity for DEN virus.

R.A. would like to acknowledge the financial support of the Medical Research Council of Canada as well as the Alberta Heritage Foundation for Medical Research for sabbatical research funds.

References


(Received 3 January 1992; Accepted 13 April 1992)