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Analysis of the Steps Involved in Dengue Virus Entry into Host Cells

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The initial steps of dengue viral entry have been divided into adsorption and penetration using acid glycine treatment to inactivate extracellular virus after attachment to baby hamster kidney (BHK) cells but prior to penetration. First, we showed that virus infection was accomplished within 2 h after adsorption. Second, the assay was used to examine the properties of dengue envelope E protein-specific monoclonal antibodies (MAbs), lectins, and heparin. We found that three MAbs, 17-2, 46-9, and 51-3, may neutralize dengue 2 virus (DEN-2) through inhibition of not only viral attachment but also of penetration. However, one MAb, 56-3.1, interfered specifically with attachment. Therefore, the functional domains of E protein involved in attachment and penetration may be different. Moreover, studies with lectins indicated that carbohydrates, especially α-mannose residues, present on the virion glycoproteins may contribute to binding and penetration of the virus into BHK and mosquito C6/36 cells. Finally, virus infectivity was inhibited by heparin through its blocking effects at both virus attachment and penetration. This suggests that cell surface heparan sulfate functions in both viral attachment and penetration of DEN-2 virus. In conclusion, our results further elucidated some aspects of the dengue virus entry process.

INTRODUCTION

Dengue virus, a member of the family *Flaviviridae,* genus Flavivirus, is an important human pathogen causing dengue fever (DF) and the more severe forms, dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (Monath, 1986). Defining how viruses interact with the cell surface is important for explaining the pathogenesis of dengue viruses. The initial events required for productive dengue infection have not been described in as much detail as for other flaviviruses, such as West Nile virus (WNV). Studies using electron microscopy have indicated that attachment is a temperature-independent process that occurs at both 4 and 37°C, whereas viral penetration proceeds only at 37°C. Penetration can occur by membrane fusion in mosquito C6/36 cells or by receptor-mediated endocytosis in monocytes (Barth, 1992; Hase *et al.,* 1989). In the presence of subneutralizing amounts of antibody, Fc receptors also mediate attachment and uptake of dengue viruses into certain target cells (e.g., monocytes and macrophages) (Gollins and Porterfield, 1985; Mady *et al.,* 1993). This entry mechanism, termed antibody-dependent enhancement (ADE), may play a role in development of DHF and DSS as a consequence of sequential infections with different dengue serotypes (reviewed in Halstead, 1988). However, ADE does not explain infection of cells without Fc receptors nor does it explain primary infection in patients lacking dengue antibody.

The viral envelope E protein of dengue virus, which is embedded in a lipid bilayer, may mediate virus attachment and penetration into cells. E protein is both a target and a modulator of the host immune response. E protein contains two sites for N-linked glycosylation; the mature glycoprotein has a molecular weight (MW) of approximately 60,000 (Smith and Wright, 1985). Use of each glycosylation site in mosquito cells appears to depend on the serotype of the virus (Johnson *et al.,* 1994). Mutations of N-linked glysosylation sites of E protein may affect virus-mediated membrane fusion and neurovirulence (Guirakhoo *et al.,* 1993; Kawano *et al.,* 1993; Sanchez and Ruiz, 1996). However, the functional significance of the attached carbohydrates themselves has not been determined.

The identification and isolation of a specific cell surface receptor for dengue viruses are important for understanding their biology. Chen *et al.* (1996b) found that recombinant E protein bound specifically to Vero, CHO, endothelial, and glial cells. In addition, this binding has been associated with heparan sulfate or glycosaminoglycans (GAGs) (Chen *et al.,* 1997). These complex charged carbohydrates are found both on the cell surface and in the extracellular matrix (Chen *et al.,* 1996b; 1997). Thus, highly sulfated heparan sulfate on the cell

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surface may serve as the initial attachment receptor for DEN-2 virus (Chen *et al.,* 1997). However, the precise mechanism of the blocking by heparin in the entry step is not clear. Additional receptors for dengue viruses may be present. For example, DEN-2 virus can infect cultured monocytes through a cellular receptor that is trypsin-sensitive; therefore, it is proteinaceous in nature (Daughaday *et al.,* 1981).

In this report, we defined the initial events of viral entry and the roles played by viral and cellular components in these processes in detail. The initial steps of dengue viral entry have been divided into adsorption and penetration by briefly exposing the virus–cell monolayers to acid glycine treatment (Highlander *et al.,* 1987; Long *et al.,* 1990). This assay was used to examine the effect of anti-E protein neutralizing antibodies on the entry process. We found that anti-E protein neutralizing antibodies may inhibit not only viral attachment but also penetration. Studies using lectins suggest that carbohydrates on the virions may play roles in dengue virus binding and penetration into BHK and mosquito C6/36 cells. Finally, we found that heparin, a related GAG, blocks virus infection at both the attachment and the penetration steps, suggesting a role for cell-surface GAGs in both processes.

RESULTS

Kinetics of viral penetration

To define more accurately the initial events of viral entry, we determined the rate of viral penetration of DEN-2 virus into baby hamster kidney (BHK) cells as described by Long *et al.* (1990). In this assay, virus, which has entered the cells, is protected from inactivation by acid treatment. Surviving viruses are capable of forming plaques. Thus, penetration is defined as the loss in sensitivity to acid inactivation. BHK cells were used as targets as they are susceptible and commonly used *in vitro* for infection by dengue viruses (Malewicz and Jenkin, 1979). Cells in 35-mm dishes were infected with DEN-2 virus (PL-046 strain) and incubated at 4°C for 2 h to allow attachment to occur (Fig. 1A). Cells were then washed and shifted to 37°C. At various time points (i.e., 0, 15, 30, 60, 90, and 120 min) after the shift, the infected cells in each dish were treated with acid glycine (pH 3.0) buffer for 1 min to inactivate extracellular virus. The acid was removed and replaced with medium and the cells were incubated at 37°C to allow plaque formation. The percentage of plaque-forming units (PFU) surviving acid treatment was calculated. At zero time, approximately 95% of the attached virus was inactivated by acid treatment (Fig. 2). As the time of incubation at 37°C prior to acid treatment increased, more virus became resistant. By 25 min, about 50% of adsorbed virus penetrated into BHK cells, and by 60 min, over 75% of the virus had penetrated the cells. By 2 h, approximately 95% of the

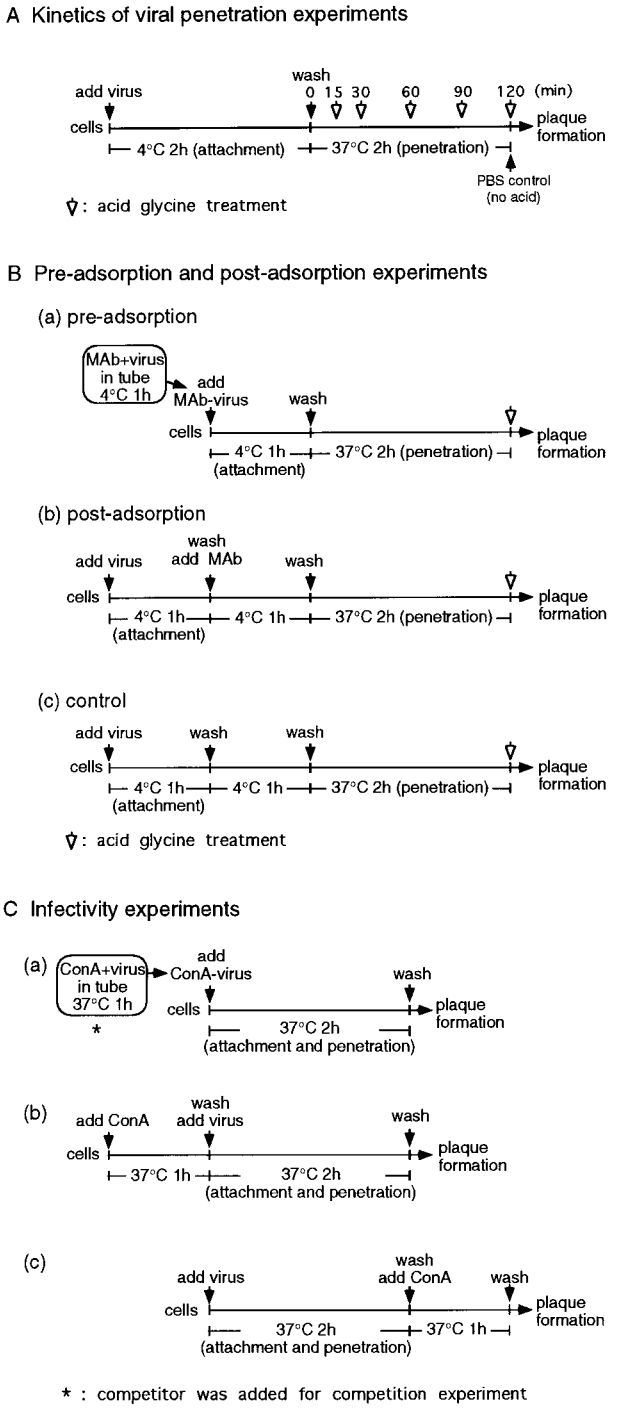

FIG. 1. Schematic representation of the strategies used in this study. (A) Kinetics of viral penetration experiments. Virus samples (100 to 250 PFU) were added to BHK cells and allowed to adsorb to cells for 2 h at 4°C. The cultures were then incubated at 37°C. At the times indicated, cells were washed with acid glycine buffer (pH 3.0) to inactivate extracellular viruses. (B) Preadsorption and postadsorption experiments for MAbs. Similar experimental procedures were also used for Con A and heparin in the same manner except that Con A and heparin were used. (C) Infectivity experiments for studying the effect of Con A on viral plaque formation. The effects of WGA, PHA-P, or heparin were also determined under similar conditions. The details are described under Materials and Methods.

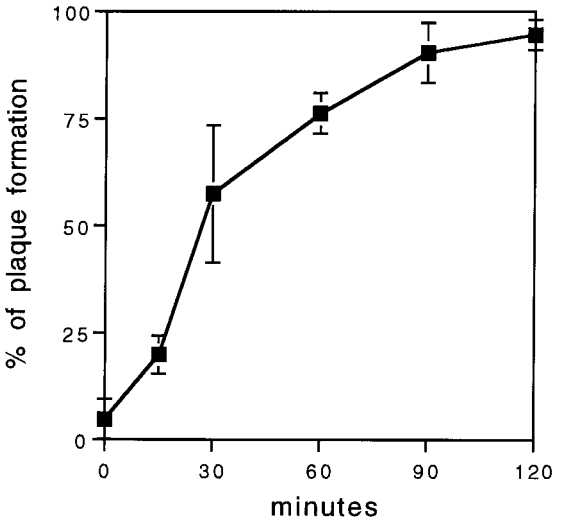

FIG. 2. Rate of penetration of DEN-2 virus. At the times indicated, cells were treated with acid glycine to inactivate extracellular viruses. The results are shown as a percentage of plaque formation when compared with controls in which PBS was substituted for acid glycine buffer.

virus had penetrated cells. In addition, the results indicated that we can easily separate entry steps into attachment and penetration using acid glycine treatment.

Neutralization of DEN-2 virus by E protein-specific monoclonal antibodies before and after virus adsorption to cell surfaces

To gain insight into the role of E protein in entry, we explored the mechanism of virus neutralization using five MAbs, 17-2, 46-9, 51-3, 55-3, and 56-3.1. These MAbs were specific for E protein of dengue virus as determined by immunoprecipitation and enzyme-linked immunosorbant assay (ELISA) (data not shown). Four MAbs, 17-2, 46-9, 51-3, and 56-3.1, were able to neutralize dengue viruses when incubated with viruses at 37°C before viral inoculation. However, MAb 55-3 was a nonneutralizing antibody (data not shown; Chen *et al.,* manuscript in preparation).

Here, this panel of MAbs were further examined by measuring their ability to neutralize virus when added before or after virus adsorption to host cells at 4°C. The entry steps were divided into adsorption and penetration using the acid glycine treatment assay as illustrated in Fig. 1B. If the mechanism of neutralization primarily involves inhibition of virus attachment, adsorbed virions should be resistant to neutralization. Alternatively, if the mechanism of neutralization involves blocking of a step in infection subsequent to adsorption, these antibodies should be able to neutralize cell surface-bound virions. Pilot studies were conducted to determine whether we could shorten the incubation time at 4°C from 2 h to 1 h. Similar results for virus penetration as shown in Fig. 2 were obtained when the virus was incubated with the cells for 1 h at 4°C (data not shown). Therefore, for the following preadsorption and postadsorption experiments, the 4°C incubation time was changed to 1 h.

Figure 3 shows the percentage of residual plaque formation for viruses treated with different MAbs added either before [Fig. 1B(a)] or after [Fig. 1B(b)] viral adsorption. First, as expected, none of the antibodies were able to enhance the plaque development of DEN-2 virus significantly at any concentration tested. The slight increase in PFU of MAb 17-2 is very low (to 110%) (Fig. 3a), and we believe that it is not significant. Second, four of the five MAbs, 17-2, 46-9, 51-3, and 56-3.1, exhibited significant neutralization titers, suggesting that their epitope specificities are localized to a site(s) that contributes to the role of E protein in virus infectivity (Figs. 3a, 3b, 3c, and 3d). Third, three MAbs, 17-2, 46-9, and 51-3, neutralized viruses not only when added before but also when added after viral attachment (Figs. 3a, 3b, and 3c). In these cases, neutralization appears to involve viral penetration, a step later than virus attachment. Interestingly, MAb 56-3.1 could neutralize viruses when added before viral adsorption, whereas it had little neutralization activity when added after adsorption (Fig. 3d). Thus, the epitope of MAb 56-3.1 may be involved mainly in viral attachment but appears not to be involved in penetration. MAb 55-3, a nonneutralizing antibody, had no effect on infectivity whether added before or after virus adsorption at 4°C (Fig. 3e). This antibody was also unable to neutralize virus infectivity when the entire incubation was carried out at 37°C (data not shown). These results indicated that the mechanism of neutralization of anti-E MAbs involved inhibition of both attachment and virus penetration into cells and strongly supports the concept that E protein plays a direct role in both aspects of the entry process of dengue viruses. It also appears that MAb 56-3.1 directly inhibits the interaction between E and its cell surface receptor and has little effect on penetration.

Determining the roles of carbohydrates of viral proteins involved in dengue entry

E protein of DEN-2 virus is glycosylated (Smith and Wright, 1985); however, the functions of the carbohydrates are not clear. To explore the possible roles of carbohydrates on viral proteins in regulating viral entry, the effects of lectins on the infectivity of DEN-2 viruses into BHK cells were first examined under three different conditions as illustrated in Fig. 1C. To examine the effects on the whole entry process, viral infection was carried out at 37°C for 2 h while attachment and penetration steps were not dissected. Viruses or BHK cells were treated with lectins including: (1) concanavalin A (Con A, which binds to α-linked terminal mannose resi-

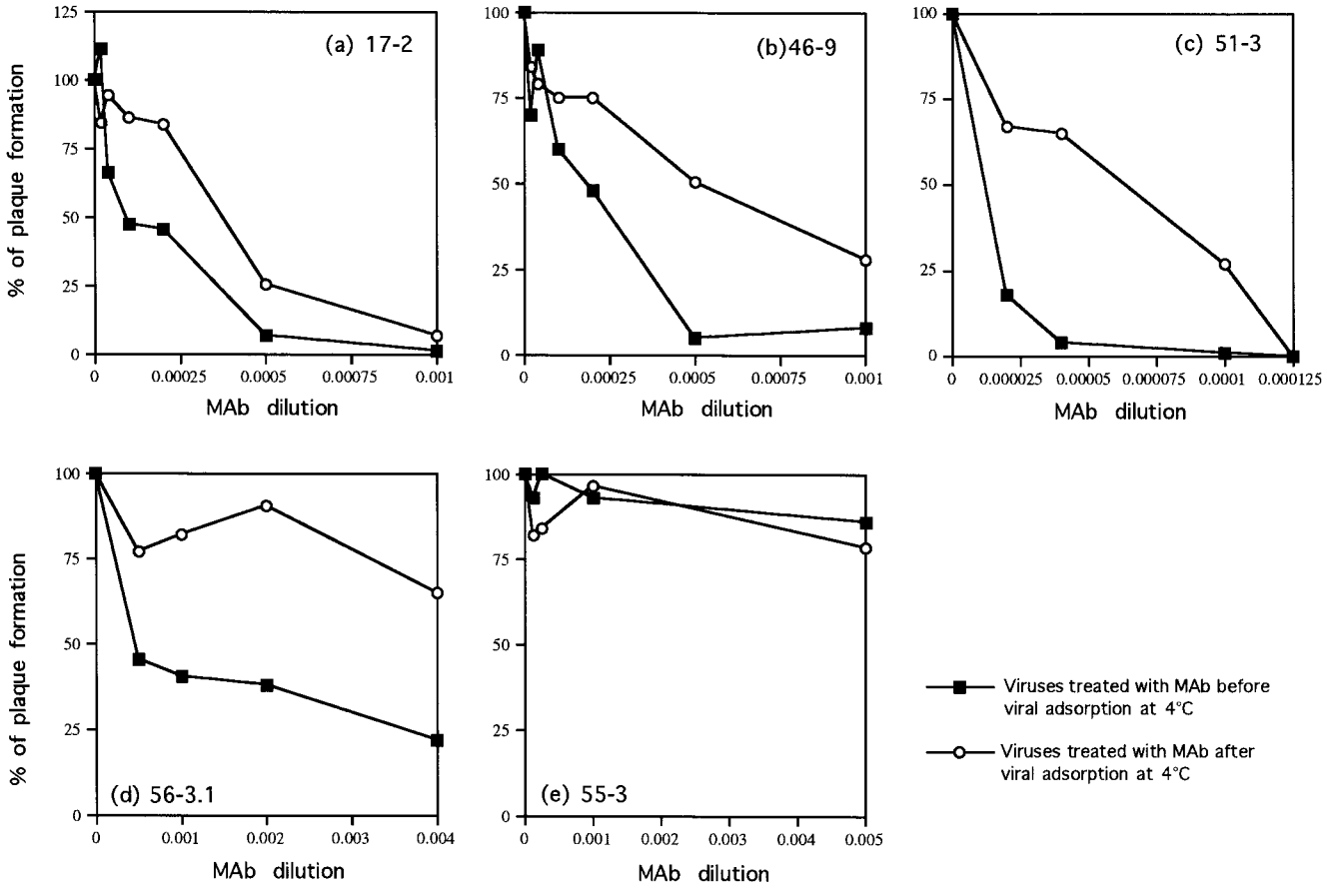

FIG. 3. Neutralization of viruses by treatment with anti-E-specific monoclonal antibodies (MAbs) before (preadsorption) or after (postadsorption) viral adsorption. DEN-2 virus was incubated with different dilutions of antibodies (a) 17-2, (b) 46-9, (c) 51-3, (d) 56-3.1, or (e) 55-3 before (■) or after (○) viral adsorption at 4°C. The results are reported as a percentage of plaque formation.

dues on N-linked high-mannose or hybrid glycans); (2) wheat germ agglutinin [WGA, which recognizes acetylglucosamine (glcNAcb1-4) on N-linked glycans]; and (3) phytohemagglutinin P (PHA-P, which recognizes oligosaccharides). The effects of the mannose-specific lectin, Con A, on plaque formation induced by DEN-2 viruses on BHK cells are shown in Fig. 4. Preincubation of virus with Con A for 1 h at 37°C prior to assessment of virus infection [Fig. 1C(a)] blocked plaque formation markedly (approximately 50% inhibition at 7.5 μg/ml) (Fig. 4a). Con A also blocked plaque formation when added to BHK cells before viral inoculation [Figs. 1C(b) and 4b]. However, there was no inhibitory effect if Con A was added to cells for 1 h after the initiation of infection [Figs. 1C(c) and 4c]. These results demonstrate that DEN-2 was inactivated when incubated with Con A during the binding of the virus and its fusion with the target cells, but that Con A had no further effect once most of the virus had penetrated. The results suggested that the carbohydrates of viral protein, probably E protein, may be required for viral infection. Alternatively, aggregates of Con A on virions may affect viral infection through steric hindrance effect. The other possibility is that Con A binding may affect the conformation of E protein, which results in malfunction of E protein. The results for the effects of WGA and PHA-P on viral infectivity are shown in Figs. 5a and 5b, respectively. Similar to the results of Con A treatment, preincubation of WGA with virus or cells before viral inoculation (but not after) greatly reduced the viral plaque formation (Fig. 5a). In contrast, there was no inhibitory effect when lectin PHA-P was used (Fig. 5b).

To confirm that the inhibitory effect of Con A was specifically due to binding to α-mannose residues of viral protein, we carried out competition experiments, using D-(+)-mannose or α-methyl-D-mannoside [Fig. 1C(a)]. Both competitors have been used successfully to compete for mannose-specific binding of Con A (Campadelli-Fiume *et al.,* 1988; DeGeorge *et al.,* 1985). The inhibitory effect of Con A was blocked by the addition of either of these competitors (Fig. 6). In addition, the α-methyl-D-mannoside competed more efficiently than D-(+)-mannose (compare Figs. 6a and 6b). The results strongly suggested that the inhibitory effects of Con A on

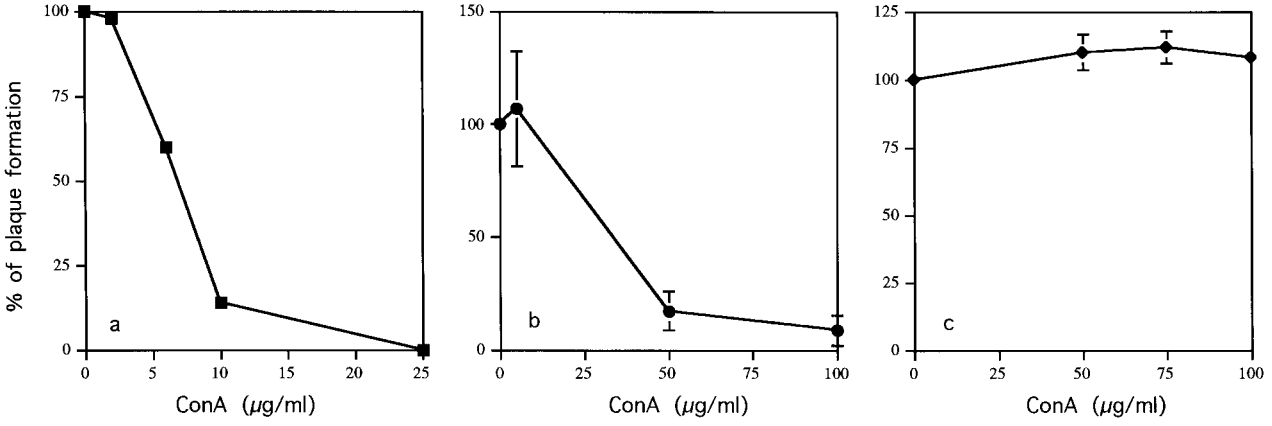

FIG. 4. Effects of Con A on plaque formation of DEN-2 virus. BHK cells were infected with DEN-2 virus under three different experimental conditions: (a) viruses treated with Con A before virus inoculation; (b) cells treated with Con A before virus inoculation; and (c) cells treated with Con A after virus infection. The results are shown as a percentage of plaque formation compared with the control reactions where infection was carried out in the absence of Con A.

viral infection were through binding to mannose residues of viral envelope proteins.

Con A inhibition at the step of attachment and penetration

To further define the mechanisms of the blocking effect of Con A, the inhibitory ability of Con A before or after viral adsorption at 4°C was evaluated. The experimental conditions were similar to those for MAb in the preadsorption and postadsorption neutralization experiment, except that Con A was used (Fig. 1B). Approximately 50% of virus was affected when 7.5–8 μg/ml of Con A was added after viral adsorption (Fig. 7). However, if added before viral adsorption, less Con A was required to obtain a similar inhibitory effect. Preincubation of virus with Con A at 4°C appeared to be more effective than preincubation at 37°C (compare Figs. 7 and 4a). In addition, Con A inhibited viral plaque formation when added after viral attachment (Fig. 7). Thus, Con A inhibits both attachment and penetration steps.

The effects of Con A on viral entry into mosquito C6/36 cells

The effects of Con A at various concentrations (1.56, 6.25, 25, and 100 μg/ml) on the infectivity of dengue

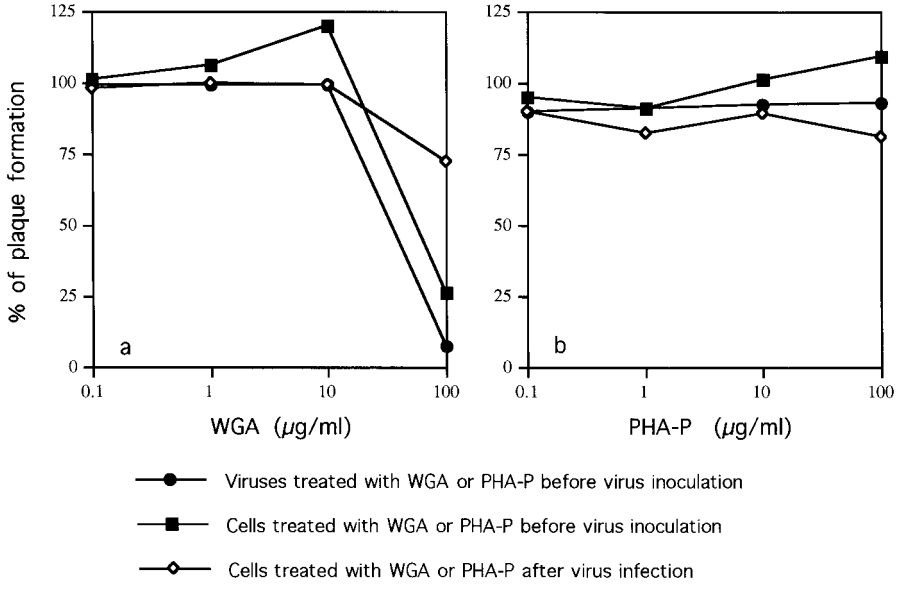

FIG. 5. Effects of (a) WGA and (b) PHA-P on infectivity of DEN-2 virus. BHK cells were infected with DEN-2 virus under three different experimental conditions: (●) viruses treated with WGA or PHA-P before virus inoculation; (■) cells treated with WGA or PHA-P before virus inoculation; and (◇) cells treated with WGA or PHA-P after virus infection. The results are shown as a percentage of plaque formation compared with the control reactions where infection was carried out in the absence of WGA or PHA-P.

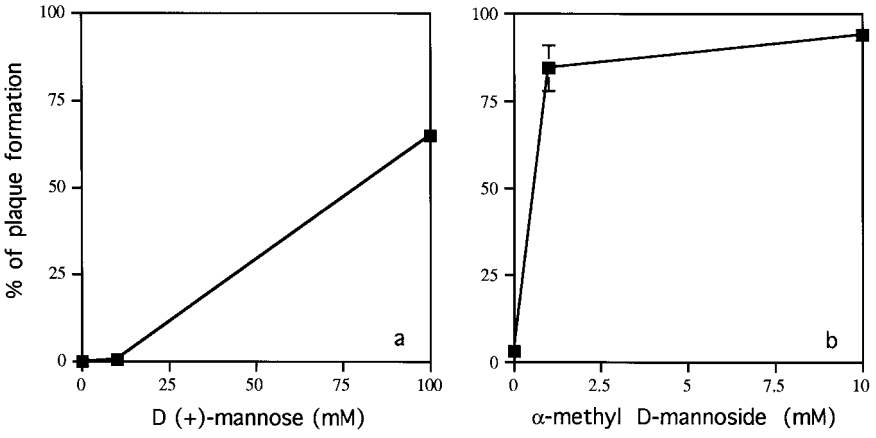

FIG. 6. Blocking the effects of Con A by competitors in the competition assay. (a) D-(+)-mannose and (b) α-methyl-D-mannoside were used as competitors to block the inhibitory effect of Con A (20 μg/ml).

viruses into mosquito C6/36 cells, natural target cells, were also examined (Fig. 8). The experimental conditions were similar, as illustrated in Fig. 1C. Since C6/36 cells did not form visible plaque after dengue infection, we utilized an immunofluorescence assay to detect the expression of viral NS1 protein. BHK cells were examined in the same way for comparison. Under the first experimental condition [Fig. 1C(a)], we found that pretreatment of viruses with Con A before viral infection affected the expression of viral antigen, NS1 (Fig. 8). Mock-infected cells showed negative fluorescence results (Figs. 8A and 8D). Preincubation of viruses with 25 (data not shown) or 100 μg/ml of Con A greatly inhibited the expression of NS1 protein in both C6/36 cells and BHK cells (Figs. 8C and 8F). Under the second experimental condition, when C6/36 or BHK cells were preincubated with 100 μg/ml of Con A before viral infection [as in Fig. 1C(b)], the inhibitory effects of Con A were also observed (data not shown). There were no obvious effects when other concentrations were used (1.65, 6.25, and 25 μg/ml; data not shown). Under the third experimental condition, when Con A was added for 1 h after the initiation of infection [Fig. 1C(c)], there was no difference for each concentration examined (data not shown). The fluorescence data were in agreement with the plaque formation data in showing that virus was inactivated when incubated with Con A during entry, but that once the virus had penetrated the C6/36 cells, Con A had no further effect. Therefore, the carbohydrates of viral protein may also be required for viral infection into mosquito cells.

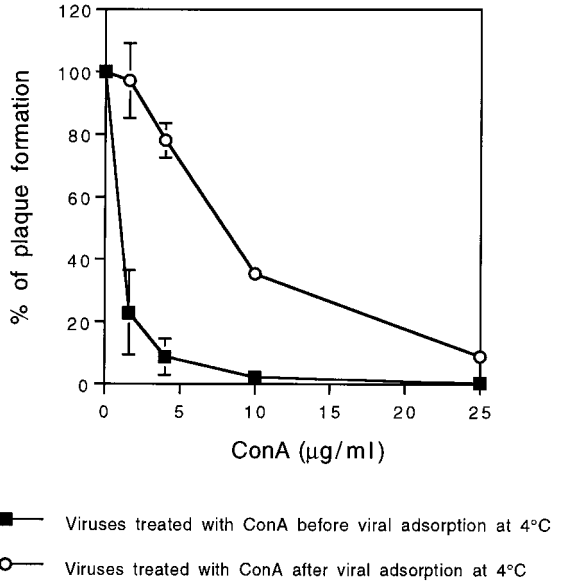

FIG. 7. Effects of Con A on preadsorption or postadsorption entry steps of DEN-2 virus. Virus was incubated with different dilutions of Con A before (■) or after (○) viral adsorption at 4°C. The results are reported as a percentage of plaque formation.

Heparin inhibition at the step of penetration

To define the precise mechanism of the blocking by heparin in the entry step, we assayed the effect of heparin on the whole entry process first without dissecting it into attachment and penetration. Similar to the lectin experiments described above, three experimental conditions were utilized (Fig. 1C). First, virus was preincubated with heparin at 37°C prior to being added to cells [Figs. 1C(a) and 9A]. Virus was inactivated in a dose-dependent manner such that approximately 50% was inactivated by incubation with 0.002 U/ml of heparin (equivalent to 0.01 μg/ml). This suggests that heparin interacts with a viral protein. There was no effect on infectivity when cells were treated with heparin before viral inoculation (Figs. 1C(b) and 9A). In addition, no inhibitory effect was observed when heparin was added after the entry events occurred [Figs. 1C(c) and 9A]. Our results suggest that heparin binds to virus and prevents the virus from interaction with a cellular component of BHK cells, sim-

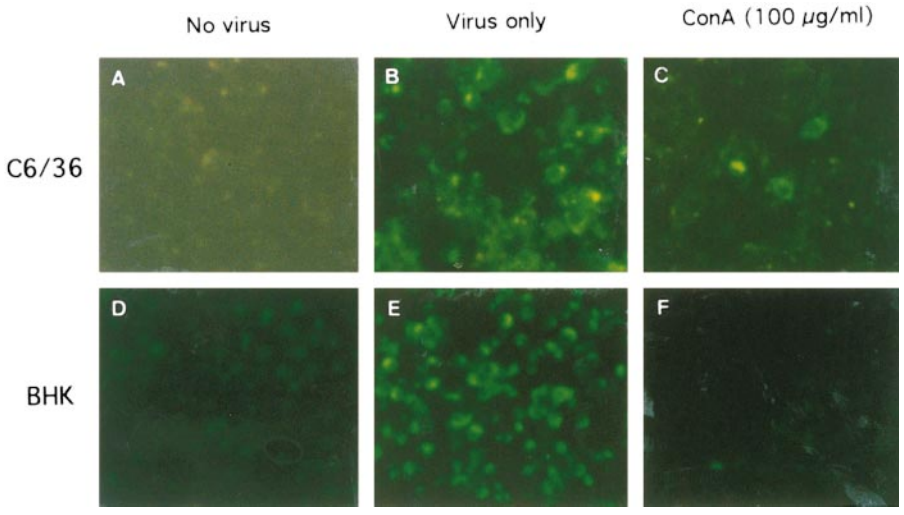

FIG. 8. Effects of Con A on infectivity of DEN-2 virus into C6/36 cells and BHK cells. Infected cells, C6/36 (A to C) and BHK cells (D to F), were tested for dengue NS1 protein expression by using a fluorescence assay. (A and D) Mock-infected cells; (B and E) cells infected with dengue viruses without Con A treatment; and (C and F) virus was incubated with 100 μg/ml of Con A before viral inoculation onto cells.

ilar to previous findings by Chen *et al.* (1997) in Vero cells.

To further explore the possible role of heparin at the penetration step, we added it before or after viral attachment at 4°C. We used acid glycine treatment to separate attachment and penetration as for MAb or Con A (Fig. 1B). We found that more heparin was required to obtain inhibition at 4°C than at 37°C (compare Figs. 9A and 9B). However, our results clearly showed that after viral attachment, heparin inhibited viral plaque formation equally efficiently when added before or after attachment (Fig. 9B). Therefore, cell

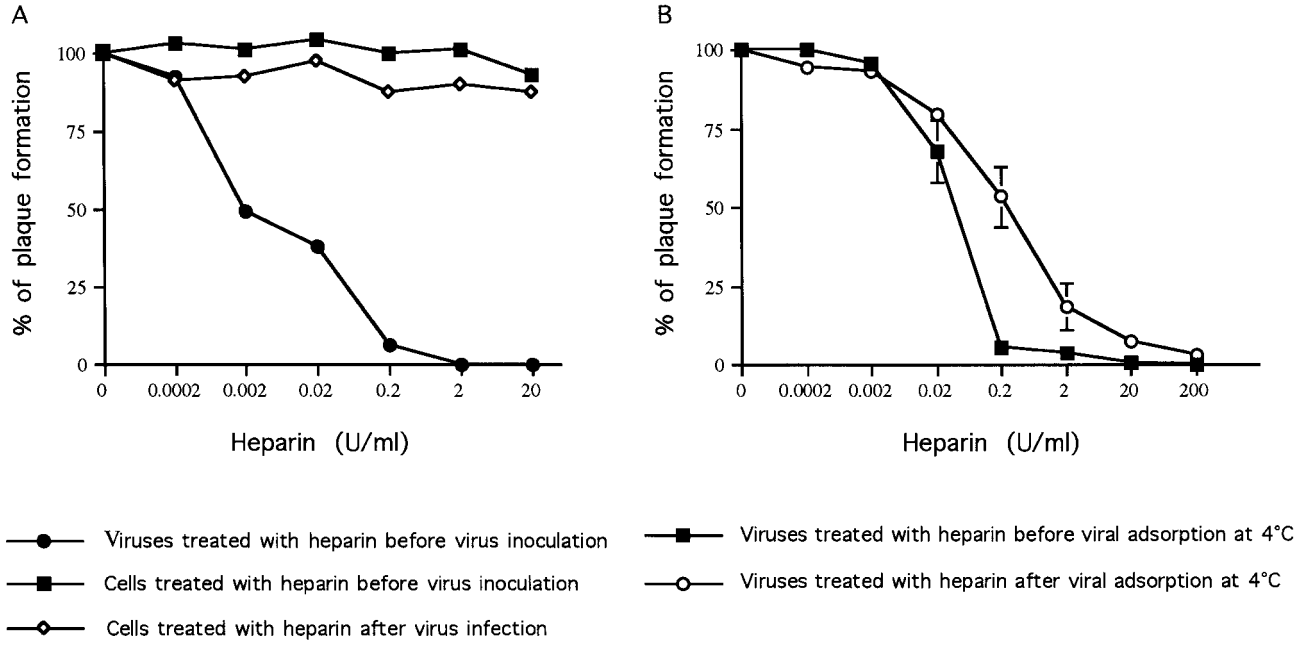

FIG. 9. Effects of heparin on the infectivity and entry steps of DEN-2 virus. (A) Effects of heparin on the infectivity of DEN-2 virus. BHK cells were infected with DEN-2 virus under three different experimental conditions as illustrated in Fig. 1C: (●) viruses were preincubated with different concentrations of heparin before virus inoculation; (■) BHK cells were treated with different concentrations of heparin before virus inoculation; and (◇) cells treated with heparin after virus infection. (B) Effects of heparin on preadsorption and postadsorption entry steps of DEN-2 viruses. The experimental conditions are illustrated in Fig. 1B. Virus was incubated with different amounts of heparin before (■) or after (○) viral adsorption at 4°C. The results are shown as a percentage of plaque formation compared with the control reactions where infection was carried out in the absence of heparin.

surface GAGs may play roles in both attachment and penetration of dengue virus.

DISCUSSION

The initial steps in viral infection are attachment and penetration into host cells. Information on the molecular mechanisms concerning these events is accumulating for several virus–host systems, although little is known concerning the molecules involved in the entry of dengue virus into the cell cytoplasm. Dengue viruses are able to attach but not penetrate into cells at 4°C (Hase *et al.,* 1989), whereas penetration requires the temperature to be raised to 37°C. Since acid glycine treatment inactivates only virus that has not penetrated into cells, we used this treatment to determine the kinetics of penetration of DEN-2 virus into BHK cells. This assay has been used to separate the attachment and penetration processes of other viruses such as herpes simplex virus (Highlander *et al.,* 1987), but this is its first application to the study of dengue virus. We found that 50% of the adsorbed virus penetrates into cells within 25 min and more than 75% of the adsorbed virus penetrates within 1 h. However, this treatment cannot distinguish between virus that has fused at the cell membrane and entered the cytoplasm and virus that may have been taken up in vesicles by endocytosis but has yet to cross the membrane boundary into the cytoplasm. In fact, both mechanisms have been reported for dengue entry (Barth, 1992; Hase *et al.,* 1989). Nevertheless, this method allowed us to easily dissect the entry steps and to further characterize the effects of antibodies and other reagents, such as lectins and heparin, on viral attachment and penetration.

Dengue E protein is an essential component for initiating infection. It mediates virus binding to cell receptors and the subsequent fusion step (Randolph and Stollar, 1990). In our study, efficient virus neutralization by three E-specific MAbs, 17-2, 46-9, and 51-3, was achieved after virus had been adsorbed to the host cell membrane. This indicated that the mechanism of neutralization also involved E protein to prevent the penetration of DEN-2 virus. This finding was consistent with the mechanism of virus neutralization that involved blocking of some step(s) after virus attachment, as previously suggested for herpes simplex virus (Highlander *et al.,* 1987). Others have reported that neutralizing antibodies against E proteins block binding of DEN-2 virus to monkey kidney (Vero) cells (He *et al.,* 1995). Interestingly, MAb 56-3.1 can neutralize viruses before viral adsorption, whereas it had little neutralization activity when added after adsorption. The results for MAb 56-3.1 suggest that the functional domains involved for attachment and penetration may be different. Further analysis of the epitopes of these antibodies will be helpful for mapping the regions on E proteins that are important for viral entry and for future vaccine development.

The E protein of dengue viruses contains two potential N-linked oligosaccharide sites. Johnson *et al.* (1994) reported that the E proteins of different serotypes (DEN-1 and DEN-2) grown in mosquito cells were heterogeneous in their utilization of potential glycosylation sites. For example, E protein of the DEN-2 virus is glycosylated only at the first site (Asn-67), whereas the E protein of DEN-1 virus is glycosylated at sites specified by Asn-67 and Asn-153 (Johnson *et al.,* 1994). In addition, glycosylation may also play roles in neurovirulence of dengue infection in mice (Pletnev *et al.,* 1993). Single amino acid substitution of Ile for Thr-155 that ablated the first conserved glycosylation sites in parental E protein of DEN-4 virus yielded a virus strain that was almost as neurovirulent as the mouse-adapted mutant (Kawano *et al.,* 1993). Thus the glycosylation site of E protein appears to play a role in neurovirulence of DEN-4 virus (Kawano *et al.,* 1993). The importance of glycosylation of envelope protein on viral entry has also been demonstrated for several other viruses. For example, variations in the number and position of N-linked glycosylation of viral hemagglutinin of influenza virus affect its receptor-binding characteristics (Deom *et al.,* 1986; Gambaryan *et al.,* 1998; Gunther *et al.,* 1993; Matrosovich *et al.,* 1997). Moreover, the *N*-glucan of the envelope protein of human immunodeficiency virus type 1 (HIV-1) is required for viral infectivity and cellular host range selection (Nakayama *et al.,* 1998).

The E proteins from virus grown in mosquito C6/36 cells are likely to contain oligosaccharides of the high-mannose type, which are able to bind to lectins including Con A and WGA (Johnson *et al.,* 1994; Smith and Wright, 1985). Our data suggested that when Con A binds to virions, their infectivity into both BHK and mosquito C6/36 cells was adversely affected at the entry step. Thus, the mannose residues of carbohydrates on viral glycoproteins may also participate in virus entry. For example, the mannose-specific lectin Con A may interfere with the direct interaction between carbohydrates of E protein and the cellular receptor(s). Alternatively, Con A may alter the conformation of the E glycoprotein and lead to inactivation. In addition, the effects of Con A may be through steric hindrance. In fact, Con A is not monovalent and may induce aggregation of virions (Gattegno *et al.,* 1992; Robinson *et al.,* 1987). Another lectin, WGA, was also found to inhibit plaque formation when added before viral inoculation. WGA is able to bind E protein; however, it does not bind as effectively as does Con A (Johnson *et al.,* 1994). This may explain why, in our experiments, more WGA than Con A on a weight basis was required for inhibition of plaque formation. There was no inhibitory effect for PHA-P. However, it is not clear whether PHA-P binds E protein or not.

Previously, it has been demonstrated that the lectin pokeweed mitogen (PWN) can enhance dengue virus infection in a mouse macrophage cell line (Hotta and Homma, 1994). Their results raised the possibility that the PWN-mediated increase in viral binding/penetration was due to the exposure of a masked dengue virus receptor (Hotta and Homma, 1994). We did not observe any enhancement with Con A, WGA, or PHA-P on viral plaque formation in BHK cells. Surprisingly, preincubation of DEN-2 virus with a low concentration of Con A (1.25 μg/ml) before but not after viral infection appeared to enhance the subsequent expression of NS1 protein in both BHK cells and mosquito C6/36 cells (data not shown). The mechanism of this enhancement is not clear, particularly since there was no significant effect on plaque formation by this concentration of Con A. Chen *et al.* (1999) found that bacterial lipopolysaccharide inhibits dengue virus infection of primary human monocytes/macrophages by blocking for virus entry via a CD14-dependent mechanism. Therefore, future studies should examine the entry events of dengue virus and the effects of various lectins on human monocytes/macrophages. Our preliminary experiments suggest that the effect of Con A on human erythroleukemia cell line K562 may indeed be different from that of BHK or mosquito cells (data not shown). Further analysis of lectins on viral entry into monocytic cells is in progress. Con A can also inhibit the infectivity of other viruses such as HIV (Lifson *et al.,* 1986; Matsui *et al.,* 1994; Pal *et al.,* 1993). The binding of mannose-specific lectins or mannose-binding protein to gp120 inhibits HIV infectivity (Pal *et al.,* 1993). Determining the mechanisms of this inhibition may be helpful for development of antiviral reagents.

Different components of host cell membranes can act as viral receptors (Haywood, 1994). The selective interaction of animal viruses with a specific cell surface receptor is an essential step in the initiation of a viral infection. This interaction often determines the host range and cellular or tissue tropism of a virus and therefore plays a key role in determining virus pathogenicity. Little is known about the full nature of the host cell receptor for dengue viruses. In part, the initial receptor for DEN-2 virus may be highly sulfated heparan sulfate (Chen *et al.,* 1997). Cell surface heparan sulfate plays a role in the attachment of several other microorganisms to mammalian cells (Rostand and Esko, 1997); the viral pathogens include herpes simplex virus (HSV) (Shieh *et al.,* 1992; Spear *et al.,* 1992; WuDunn and Spear, 1989), HIV-1 (Patel *et al.,* 1993), type O foot-and-mouth disease virus (Jackson *et al.,* 1996), respiratory syncytial virus (Krusat and Streckert, 1997), vaccinia virus (Chung *et al.,* 1998), adeno-associated virus (Summerford and Samulski, 1998), and Sindbis virus (Byrnes and Griffin, 1998; Klimstra *et al.,* 1998). However, additional receptors may be required for binding and entry of these viruses (Montgomery *et al.,* 1996). Our results from heparin blocking experiments further suggest that interaction between dengue virus and cell surface GAGs is also important for penetration. Specifically, the binding of heparin to the E protein of DEN-2 virus may alter the ability of E protein to function in both attachment and penetration. Recently, Laquerre *et al.* (1998) showed that HSV binding to the heparan sulfate receptor is also required in the process of virus penetration. Heparan sulfate may be required to increase binding to a second receptor potentially recognized by viral envelope protein or to initiate changes in the envelope of adsorbed virion that trigger the process of envelope fusion with the cellular membrane.

This report addresses interactions between viruses and cells with respect to viral entry. Using the acid glycine method, we demonstrated that both attachment and penetration steps can be affected by three neutralizing MAb, Con A, or heparin. This assay provided a useful approach in the further study of dengue virus. Whether the same receptor or entry mechanism is utilized in all serotypes of dengue viruses or in all tissues infected by the viruses needs further investigation. Indeed, various cell lines vary in their susceptibility to DEN-4 infection and may be determined largely by the presence of a cell receptor capable of binding dengue viral E protein (Anderson *et al.,* 1992). Our results will be valuable for understanding the interactions of dengue viruses with the cell surface. Future research on defining how viral proteins interact with the cell surface will help clarify the pathogenesis of dengue viruses and facilitate the design of antiviral agents that can interfere early in viral infection.

MATERIALS AND METHODS

Cell cultures and virus preparation

Aedes albopictus C6/36 cells were grown at 28°C to confluency in half Dulbecco's modified minimum essential medium (DMEM) and half Mitsuhashi and Moramorosch insect medium (M & M), supplemented with 10% fetal calf serum (FCS, Biological Industries) and antibiotics. BHK cells were propagated in RPMI 1640 medium (Gibco) supplemented with 5% FCS and antibiotics. DEN-2 virus (PL-046 strain), a Taiwanese strain obtained from one dengue fever patient in 1987, was kindly provided by the National Institute of Preventive Medicine, Taipei, Taiwan, R.O.C. Viruses were propagated in C6/36 cells and titered on BHK cells at 37°C.

Viral penetration assay

The rate of viral penetration was determined by inactivation of extracellular viruses with a low-pH glycine buffer as used by Long *et al.* (1990) (Fig. 1A). Briefly, BHK cells in 35-mm-diameter tissue culture dishes were in-

oculated with 100 to 250 PFU of virus and incubated at 4°C with gentle rocking for 2 h. Unbound virus was removed by two washes with ice-cold phosphate-buffered saline (PBS; pH 7.5). The dishes were overlaid with medium and shifted to 37°C. At various times after the temperature shift, the infected cells were treated with acid glycine (pH 3.0) solution (8 g of NaCl, 0.38 g of KCl, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 7.5 g of glycine/L, pH adjusted to 3 with HCl) (Cai *et al.,* 1988) for 1 min at room temperature (RT) to inactivate extracellular viruses and then washed twice with PBS and overlaid with 1% agarose–1.25% FCS–RPMI medium before further incubation at 37°C for 7 days. Infected monolayers were then fixed with formalin and stained with crystal violet. Plaques, representing intracellular acid-resistant viruses, were counted, and the percentage of PFU surviving acid treatment was calculated using the following formula: 100 × number of PFU (acid treated)/number of PFU (PBS control). The number of plaques formed on cells without acid glycine treatment (PBS control) was considered as 100%. Experiments were performed in duplicate for at least two independent experiments and the plaque numbers were averaged.

Monoclonal antibodies to E and NS1 proteins

The procedure for immunization and production of hybridoma cell lines secreting dengue E or NS1 protein-specific monoclonal antibodies (MAbs) has been described in detail elsewhere (Chen *et al.,* 1996a; Lin *et al.,* 1998). The hybridoma cell lines secreting specific antibodies were identified by ELISA and immunoprecipitation assay with DEN-2-infected C6/36 cell lysates as previously described (Chen *et al.,* 1996a; Lin *et al.,* 1998). The MAbs against DEN-2 E protein were 17-2 (Lin *et al.,* 1998), 46-9, 51-3, 55-3, and 56-3.1 (Lin *et al.,* 1998). The MAb 8-1 was specific for NS1 protein (Chen *et al.,* 1996a).

Preadsorption and postadsorption virus neutralization experiments

BHK cells were used as target cells instead of cells bearing Fc receptors such as monocytic cells, since antibody-dependent enhancement will make the results with antibodies more difficult to interpret. Pilot studies were conducted to determine whether we could shorten the incubation time at 4°C from 2 h to 1 h. Similar results for virus penetration were obtained when the virus was incubated with the cells for 1 h at 4°C. Therefore, for the following preadsorption and postadsorption experiments, the 4°C incubation time was changed to 1 h. For preadsorption virus neutralization, virus (100 to 250 PFU) was preincubated with various dilutions of individual anti-E MAbs for 1 h at 4°C and then added to BHK cell monolayers in six-well trays and incubated at 4°C for 1 h [Fig. 1B(a)]. For postadsorption virus neutralization experiments, virus samples were added directly to the monolayers for 1 h at 4°C [Fig. 1B(b)]. Unadsorbed virus was removed by washing the cells, and bound viruses were incubated with various dilutions of individual MAbs for an additional 1 h at 4°C [Fig. 1B(b)]. The control set of virus samples was plated for 1 h at 4°C, washed, and incubated for an additional 1 h at 4°C [Fig. 1B(c)]. The cells were then washed twice and incubated for an additional 2 h at 37°C under 5% FCS–RPMI to allow penetration to occur. After 2 h at 37°C, virus that had not penetrated was inactivated by the acid glycine (pH 3.0) solution. Cells were then overlaid with 1% agarose–1.25% FCS–RPMI. Plaque formation was determined on day 7. The results are expressed as the percentage of plaques formed in the presence of antibody, that is, the number of PFU obtained with antibody treatment [Figs. 1B(a) and (b)] compared with the value for a control sample of virus incubated without antibody [Fig. 1B(c)].

Treatment of cells with lectins or heparin

Lectins used were Con A (Sigma Chemical Co.), wheat germ agglutinin (WGA) from *Triticum vulgaris* (Sigma Chemical Co.), and phytohemagglutinin PHA-P from *Phaseolus vulgaris* (Sigma Chemical Co.). Heparin, a heparan sulfate-like molecule, was purchased from Sigma Chemical Co. One milligram of heparin contains approximate 170 units of activity. BHK cell monolayers in six-well tissue culture plates were infected with 0.4 ml (100 to 250 PFU) of virus inoculum under three different experimental conditions. The infectivity experiments examined the effects of various chemicals on the whole entry process. First, virus was preincubated with different concentrations of individual chemicals for 1 h at 37°C [Fig. 1C(a)]. The mixture was then added to BHK cells and incubated for 2 h at 37°C for viral entry (both attachment and penetration) to occur. For the second set of treatments, BHK cells were incubated with various amounts of chemicals at 37°C for 1 h and washed twice before virus inoculation [Fig. 1C(b)]. The treated cells were then infected. For the third set, BHK cells were incubated with virus at 37°C for 2 h, the unbound virus was removed, and the cells were then treated with various amounts of chemicals at 37°C for 1 h [Fig. 1C(c)]. In each case, infected cells were then washed and overlaid with 1.25% FCS–RPMI containing 1% agarose. Plaque formation was determined on day 7. The results are shown as a percentage of plaque formation (plus chemicals) [Figs. 1C(a), (b), and (c)] compared with the individual control reactions where infection was carried out in the absence of chemicals.

The effects of Con A and heparin on viral penetration were also analyzed as preadsorption and postadsorption experiments as described above for viral neutralization

experiments (Fig. 1B), except that MAb was replaced with Con A or heparin. The results were expressed as the percentage of plaque formation, that is, the number of PFU obtained with treatment compared with the value for a control sample of virus incubated without Con A or heparin.

Competition assays

D-(+)-Mannose and α-methyl-D-mannoside (Sigma Chemical Co.) were used as competitors to block the inhibitory effects of Con A. In the presence of 20 μg/ml of Con A, 100 to 250 PFU were mixed with different amounts of each competitor and incubated for 37°C for 1 h [Fig. 1C(a)]. The viral mixtures were added to BHK cells and incubated for 2 h at 37°C. Infected cells were then washed and overlaid with 1% agarose–1.25% FCS–RPMI. The percentage of plaque formation was calculated as follows: 100 × [(the number of PFU in the presence of Con A and competitor)/(the number of PFU in the absence of Con A and competitor)]. The number of PFU formed in the absence of both Con A and competitor was considered 100%, whereas the number of PFU formed in the presence of Con A only was 0%.

Immunofluorescence assay

C6/36 and BHK cell monolayers in 24-well tissue culture plates were infected with virus (m.o.i. of 1) under three different experimental conditions as described in Fig. 1C. The immunofluorescence assay, instead of plaque assay, was conducted to detect the infected C6/36 cells, since C6/36 cells did not form obvious plaque. In addition, C6/36 cells and BHK cells were incubated at 28 and 37°C, respectively. At 4 days postinfection, the infected cells were examined by indirect immunofluorescence. Cells were fixed with 80% acetone in PBS for 10 min and then saturated with 5% dry milk in PBS for 1 h at RT or overnight at 4°C. The fixed cells were incubated with anti-NS1 MAb 8-1 (1:1000) at 37°C for 1 h and then incubated with goat anti-mouse IgG conjugated to fluorescein isothiocyanate isomer (GAM–FITC) (1:500) at 37°C for 1 h. Between each step, the infected cells were washed twice with PBS. Microscopy was performed with a Nikon fluorescence inverted microscope. Experiments were performed in duplicate for three independent experiments.

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