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# Infection of cells by Sindbis virus at low temperature

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**Abstract**

Sindbis virus, which belongs to the family *Togaviridae* genus *Alphavirus* infects a variety of vertebrate and invertebrate cells. The initial steps of Sindbis virus infection involve attachment, penetration and uncoating. Two different pathways of infection have been proposed for Alphaviruses. One proposed mechanism involves receptor mediated virion endocytosis followed by membrane fusion triggered by endosome acidification. This virus–host membrane fusion model, well established by influenza virus, has been applied to other unrelated membrane-containing viruses including Alphaviruses. The other mechanism proposes direct penetration of the cell plasma membrane by the virus glycoproteins in the absence of membrane fusion. This alternate model is supported by both ultrastructural [Paredes, A.M., Ferreira, D., Horton, M., Saad, A., Tsuruta, H., Johnston, R., Klimstra, W., Ryman, K., Hernandez, R., Chiu, W., Brown, D.T., 2004. Conformational changes in Sindbis virions resulting from exposure to low pH and interactions with cells suggest that cell penetration may occur at the cell surface in the absence of membrane fusion. Virology 324(2), 373–386] and biochemical [Koschinski, A., Wengler, G., Wengler, G., and Repp, H., 2005. Rare earth ions block the ion pores generated by the class II fusion proteins of alphaviruses and allow analysis of the biological functions of these pores. J. Gen. Virol. 86(Pt. 12), 3311–3320] studies. We have examined the ability of Sindbis virus to infect Baby Hamster Kidney (BHK) cells at temperatures which block endocytosis. We have found that under these conditions Sindbis virus infects cells in a temperature- and time-dependent fashion.


*Keywords:* Alphaviruses; Cell penetration; Membrane fusion

## Introduction

It is widely held that membrane-containing viruses employ those membranes as tools of infection (Eckert and Kim, 2001; Marsh and Helenius, 2006). These enveloped viruses gain entry to potential host cells by fusing the virus membrane with the host cell membrane. This fusion event is mediated by proteins integrated into the virus membrane which serve as motors that drive the fusion process (Jardetzky and Lamb, 2004). Virus fusion proteins can mediate the process of fusion with a host cell membrane in a low pH-dependent or -independent manner (Marsh and Helenius, 2006). In the low pH-independent pathway, it is proposed that virus glycoproteins interact with a cell receptor and that this interaction induces protein conformational changes which drive the fusion of the virus membrane with the cell membrane. An example of a virus which enters cells by fusion at the cell surface is HIV (Cardoso et al., 2005; Eckert and Kim, 2001). In the low pH-dependent pathway it is proposed that upon interaction with a receptor, virus particles are internalized into endosomes which upon acidification, induce conformational changes in the virus proteins which lead to membrane fusion (Kielian and Jungerwirth, 1990). This latter pathway has been proposed for many families of viruses and is best demonstrated for influenza virus (Stegmann et al., 1987). Influenza virus has been convincingly demonstrated to enter cells from the low pH environment of a late endosome (Tamm, 2003). The process of influenza virus fusion with the cell membrane has been investigated using a liposome model system (Stegmann et al., 1985). It has also been demonstrated that alpha (and flavi) viruses can fuse with protein-free liposome membranes upon exposure to acidic pH (Smit et al., 1999). A significant difference between the putative fusion inducing proteins of influenza virus and the alphaviruses is the

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dramatic dissimilarity in their structure and sequence (Gibbons et al., 2004; Modis et al., 2004; Zaitseva et al., 2005). Because of these differences these proteins are referred to as Type I (Flu) and Type II (Alphavirus) fusion proteins. Type I fusion proteins are found in a wide variety of unrelated virus families (Eckert and Kim, 2001). Type II fusion proteins are found only in the insect vectored Alpha and Flaviviruses. The ability of SFV and Sindbis virus to fuse with liposomes and the observations that inhibitors of endosome acidification or mutations which block endosome formation can prevent subsequent virus RNA or protein synthesis has been presented as evidence that Alpha and Flaviviruses also penetrate cells from the acidic environment of endosomes (DeTulleo and Kirchhausen, 1998; Helenius et al., 1982). Each of these experiments has presented problems in the interpretation of the data. Fusion with protein-free liposomes has been demonstrated to "absolutely" require the presence of cholesterol in the liposome membrane (Kielian and Helenius, 1984). Liposomes employed in these studies typically contained 25–50 mol% cholesterol. Insects are the vector for these viruses and their membranes have less than 1% cholesterol (Rietveld et al., 1999). Insect cell lines, such as SF21 cells (Cha et al., 1997) (Invitrogen, Carlsbad, CA), have been adapted to synthetic growth medium (Weiss et al., 1993) (SF900IISFM; Gibco, Invitrogen) that is very low in cholesterol. It has been shown that Japanese Encephalitis Virus will infect and replicate in these cells (Kim et al., 2004). We have found that Sindbis virus readily infects and can be passaged in these cells to high titers (to be published elsewhere). Studies with inhibitors of endosome acidification or cells encoding genetic defects in the endocytic pathway measure the lack of products of late events such as RNA or protein synthesis to determine that penetration has not taken place. These are events which occur several steps after penetration is completed and raise the possibility that the defect in virus production may occur after penetration. In one instance it has been shown that an agent blocking endosome acidification does not block penetration but rather blocks processing of virus nonstructural proteins to form the RNA replication complex (Hernandez et al., 2001).

The structural organization of the Alpha and Flaviviruses presents an innate barrier to the fusion process. The Alphavirus particle is composed of two symmetrically identical $T=4$ icosahedral protein shells nested one inside the other (Paredes et al., 1993). The virus membrane is sandwiched between the two shells and protected by the outer shell. The lateral associations which stabilize the outer protein shell are, in turn, stabilized by intramolecular disulfide bridges (Anthony et al., 1992; Mulvey and Brown, 1994). The E1 protein is assembled in a lateral matrix with fenestrations at the 5- and 6-fold axes and is located directly above the membrane bilayer. The disassembly of this E1 protein lattice would be required to expose the underlying membrane bilayer and allow the fusion event to occur. We have previously shown that the process of fusion of the Sindbis virus membrane with the membrane of a living host cell is a two-step process requiring exposure to acidic pH (to set up conditions for fusion) and return to neutral pH (to execute the fusion event) (Edwards and Brown, 1986; Paredes et al., 2004). This contrasts with the process of virus fusion with artificial liposomes which does not require a return to neutral pH and suggests that interactions with living cells are more complex in nature. Electron cryo-microscopy has revealed a massive reorganization of the surface of Sindbis virus as the pH is lowered to 5.3 (Paredes et al., 2004). This reorganization renders the particles insoluble and exposes a new protruding structure at the center of the strict 5-fold axis. At this pH Sindbis fuses with protein-free liposomes (Smit et al., 1999). This is likely due to the interaction of newly exposed hydrophobic protein domains within protein E1 (Paredes et al., 2004). This interaction combined with the chemical composition of the liposome membrane and its extreme curvature may physically disrupt the protein–protein interaction in the envelope glycoproteins allowing fusion to occur. When Sindbis virus interacts with the receptor of a living cell the reorganization of the surface seen at acid pH is not adequate to induce fusion (Edwards and Brown, 1986; Paredes et al., 2004). Electron cryo-microscopy of virions shows that as virus is returned from acid pH to neutral pH the surface of the virus undergoes a second set of conformational changes in which the structure protruding at the 5-fold axis is withdrawn into the virus structure and large fissures develop in the virus surface along the 2-fold axis (Paredes et al., 2004). These changes may compromise the structural integrity of the virion to such an extent that fusion may now take place.

In electron microscope studies, we have found that when infectious Sindbis virus particles interact with the surface of living cells, they lose their electron-dense core suggesting that the virus RNA has been transferred to the cell (Fig. 1) (Paredes et al., 2004). Events related to the process of penetration occurred at the cell surface in the absence of endocytosis and at neutral pH. A similar result has been reported for the interaction of Dengue virus and Japanese Encephalitis virus with host cells (Hase et al., 1989). These data led to the hypothesis that

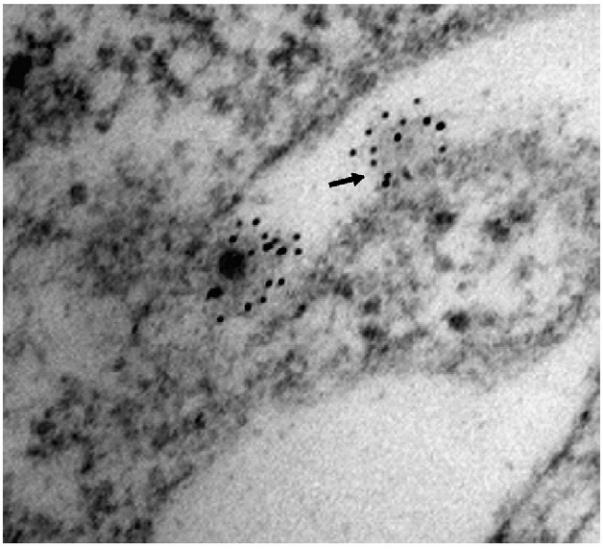

Fig. 1. Sindbis virions attached to the surface of a BHK cell. Virus was absorbed to cells as described in the text. After attachment virus proteins were immunolabeled with anti Sindbis serum and secondarily labeled with anti IgG serum conjugated with gold beads. One virion is seen to contain its electron dense core the other (arrow) appears empty. It is of note that both particles bind the antibody.

interaction of the virus spike glycoproteins with the cell surface resulted in the formation of a protein pore in the cell plasma membrane through which the virus RNA passed to initiate infection (Paredes et al., 2004). Evidence supporting the formation of such a pore during virus infection has subsequently been provided by other investigators (Koschinski et al., 2005). In the experiments presented therein (Paredes et al., 2004), we found that about 25% of particles attached to the cell surface became empty at 37 °C. To our surprise 3–4% of particles attached became empty if the cell monolayers were maintained at 4 °C. This number is significant because Sindbis virus does not assemble empty particles, the virus employed was purified 2X by density gradient centrifugation and no empty particles were seen if virus was fixed prior to addition to cells. These observations suggested that some events related to the infection process could occur at temperatures which prevent endocytosis and indeed prevent all vesicular transport (Lippincott-Schwartz et al., 2000).

## Results

### *Interaction of Sindbis virus with cells at low temperature*

It is well known that at temperatures below 19 °C endocytosis in mammalian cells is arrested and at temperatures around 15 °C all cellular vesicular transport is stopped. This gentle technique has been utilized by cell biologists to study aspects of vesicular transport in living cells (Lippincott-Schwartz et al., 2000). To further characterize the interaction of Sindbis virus with cells under conditions which block endocytosis we examined the ability of the virus to establish infection at 5 °C and 15 °C. Our approach was to create an antibody escape experiment in combination with a standard plaque assay. In this experiment we measured the amount of virus which had penetrated cells by determining the amount of cell associated virus which escaped inactivation by added antibody at low temperature. We first determined the ability of the antibody employed in this study to bind to virus after the process of infection was completed. This was done by attaching virus to cells at 5 °C for 15 min. The cells were then washed at 5 °C to remove unattached virus and were then placed at 22 °C for 1 h. The virus cell complex was fixed for electron microscopy as described in Methods and exposed to anti-virus serum followed by the addition of gold-conjugated anti-IgG antibody. A representative electron micrograph of a thin section of virus cell complexes prepared by this protocol is shown in Fig. 1. This image shows that the antibody can bind to cell associated viruses which have or have not lost their electron-dense core. This experiment suggests that the complex conformational changes accompanying cell penetration do not eliminate antibody binding.

The antibody described above was employed under identical conditions in an antibody escape experiment in the context of a standard plaque assay. The protocol consisted of the following steps: 1. Serial dilutions of infectious virus were bound to identical monolayers of cells at 5 °C for 15 min (attachment). 2. The cells were washed to remove unbound virus and placed in media at either 5 °C, 15 °C or 22 °C for either 0 min (control) 30 min or 60 min (infection/penetration). 3. The cells were then washed and incubated at 5 °C in media containing 0.04 mg/ml anti-SVHR whole virus IgG (the same antibody used in Fig. 1) for 60 min (*to inactivate virus particles* which had not infected the cell or had not undergone any conformational change leading to infection which would place virus beyond antibody inactivation) or in medium without antiserum. 4. The monolayers were overlaid with agarose, incubated at 37 °C for 36 h and stained with neutral red as in a standard plaque assay (Renz and Brown, 1976). In order to interpret the result of this experiment it was necessary to demonstrate that the antiserum employed could inactivate virus particles after the virus had attached to the cell surface. This was done by following the protocol presented above but eliminating step 2, the penetration step. In this control experiment we found that 13.9% of the virus attached to the monolayer formed plaques after step 3 (Fig. 2). This suggests that 86.1% of virus attached to cells at 5 °C for 15 min is inactivated by the antiserum treatment and sets the baseline for experiments examining infection at 5, 15 and 22 °C. The data from these experiments are presented in Fig. 2. The result for each condition (incubation times and temperatures) is plotted as the percentage of the number of plaques formed by the virus stock in an identical experiment where antibody-free media was used in step 3 of the assay (step 3 described above). Thus, the plotted percentage reflects the fraction of virus that escape inactivation by the virus during the incubation using the indicated times and temperatures. Each entry is the average of three independent experiments. We found that significant numbers of virus particles could establish infection at low temperatures and that the overall levels of virus infection increased as temperature was increased from 5 °C to 15 °C and as time was increased from 30 min to 60 min. A significant fraction of virions is also capable of initiating infection at 15 °C in 30 min (57.8%) and 60 min (68.0%). Even when the

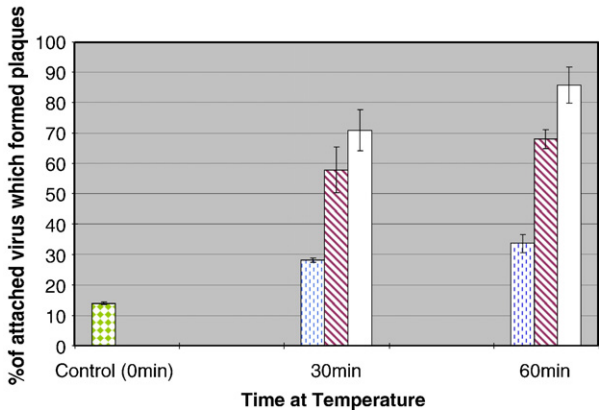


Fig. 2. Formation of infectious centers by Sindbis virus at low temperature. Cells were infected with Sindbis, washed and exposed to antiserum as described in the text. The amount of virus which was able to infect cells under the conditions of time and temperature indicated was determined as the percentage of the total number of plaque forming units applied to the cell monolayers (see text). The 0 time point at 5 °C is the control for the ability of the antiserum to inactivate virus after attachment to cells. Checkered: control. Squares: 5 °C. Chevron: 15 °C. White: 22 °C. Each entry is the average of three independent experiments.

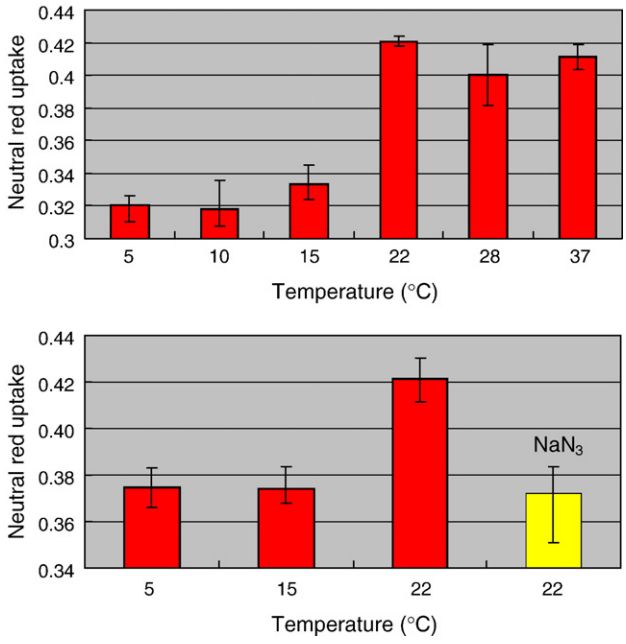


Fig. 3. Endocytosis by BHK cells at cold temperatures and warm temperatures. (A) Cells were measured for their ability to incorporate neutral red as described in the text at various temperatures. (B) The ability to incorporate neutral at low temperature is compared to the ability to incorporate neutral red in cells at 22 °C treated or not treated with sodium azide. Each entry represents the average of 3 independent experiments.

incubation is done at 5 °C we can detect viral infection in 28.2% and 33.7% of the virions, at 30 and 60 min, respectively. These experiments were also conducted with antibody and complement in the inactivation step (data not shown). The addition of complement did not change the outcome of these experiments. These results indicate that events leading to the infection of cells can occur at temperatures which do not permit endocytosis and that this process occurred in a time- and temperature-dependent fashion yielding measurable kinetics of the infection reaction.

*Endocytosis by BHK cells at low temperature*

The results presented in Fig. 2 could alternatively be explained by the possibility that endocytosis is not blocked but rather is slowed as temperature is lowered. While the effects of temperature on endocytosis have been extensively studied, and no endocytosis is seen to occur at 15 °C this parameter was measured in a standard assay of endocytosis which employs uptake of the vital dye neutral red (Long et al., 2005; Weeks et al., 1987). Neutral red is a large molecule that can be transported into living cells only by endocytosis. Equivalent monolayers of cells were treated with 0.00025% neutral red solution at the temperatures specified, for 30 min. The dyed cells were subsequently washed with cold buffer and the neutral red was extracted from the cells with organic solvent (1:1 acetic acid/ethanol). The amount of cell associated neutral red was determined by measuring absorbance of the extract at 550 nm. The results of these experiments are presented in Fig. 3 and each entry is the average of three independent experiments. We found that at low temperatures (5, 10 and 15 °C) a low amount of neutral red was cell associated. This amount remained constant in all three of the low temperatures tested. At temperatures of 22 °C and higher a much greater amount of cell associated neutral red was recovered. A statistical analysis of the data (see Methods) gave a *p*-value of less than 0.0001 of the data from all six temperatures, which affirms a 99.99% confidence that the shift of neutral red uptake was not due to variance in the experiment. The same statistical procedure was applied to the three groups of neutral red uptake data. Data from 5, 10 and 15 °C experiments resulted in a *p*-value of 0.3664, while 22, 28

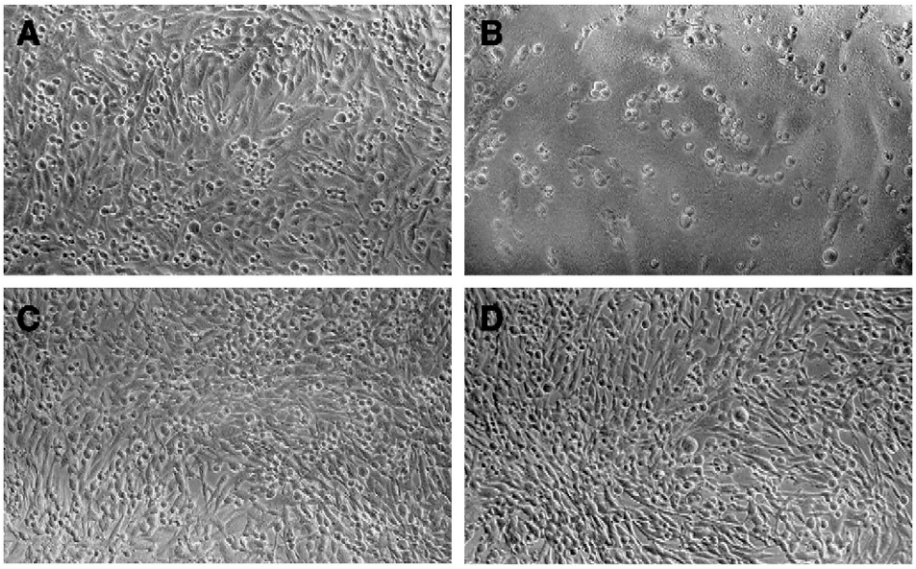


Fig. 4. Sindbis virus mediated cell–cell fusion at 37 °C and 15 °C. Cell fusion was conducted as described in the text. (A) Virus cell complexes maintained at pH 7.2 at 37 °C. (B) Virus cell complexes after exposure to pH 5.3 and returned to pH 7.2 at 37 °C. (C) Virus cell complexes maintained at pH 7.2 at 15 °C. (D) Virus cell complexes after exposure to pH 5.3 and returned to pH 7.2 at 15 °C.

and 37 °C gave a *p*-value of 0.2027. Thus, there is no significant difference among the three groups of data either below or above 15 °C. These data are in agreement with the observation that endocytosis is efficient at temperatures of 22 °C and higher but raised the possibility that lower temperatures slowed but did not arrest the process of endocytosis. To examine this possibility we repeated the neutral red uptake experiment at 5, 15 and 22 °C and included an experiment with cells at 22 °C which had been treated with 0.1 M sodium azide for 30 min prior to the addition of neutral red. This treatment killed all cells after a 30-min treatment as measured by trypan blue uptake.

After 30 min the amount of neutral red found associated with the azide treated cells was equivalent to that associated with the 5 and 15 °C cells but well below the 22 °C cells not treated with azide. These data suggest that the neutral red recovered from the low temperature cells is due to background association and not to endocytosis. These data support the previous investigations demonstrating that endocytosis in mammalian cells does not take place at 15 °C and below (Lippincott-Schwartz et al., 2000).

*Sindbis virus mediated cell–cell fusion at low temperature*

The data presented above suggest that events leading to the infection of living cells by Sindbis virus can occur in the absence of endocytosis. We have proposed that these events also occur without the need for membrane fusion (Paredes et al., 2004). To further examine this possibility we have determined if the phenomenon of Sindbis virus mediated cell–cell fusion can take place at low temperatures. We have previously shown that in contrast to fusion of virus with artificial liposomes, fusion of wild type virus with living cells does not occur at acidic pH but rather requires brief exposure to acidic pH (to establish conditions for fusion) followed by return to neutral pH (Edwards and Brown, 1986; Paredes et al., 2004). We examined the ability of Sindbis virus to fuse BHK cells at 15 and 37 °C using standard procedures described previously (Edwards and Brown, 1986; Paredes et al., 2004) and in Methods. The result of this experiment is presented in Fig. 4. We found that at 15 °C no evidence of cell fusion could be detected even if cells were incubated for several hours. At 37 °C significant fusion of the cell monolayer was seen. This result implies that infection can take place under conditions which prevent membrane fusion.

## Discussion

The use of low temperatures to block endocytosis and other events related to vesicular transport has been widely employed by cell biologists to study these processes (Lippincott-Schwartz et al., 2000). These studies have established that the process of endocytosis does not occur at temperatures below 19 °C. We have used the benign treatment of lowering the incubation temperature to establish conditions for studying the role that vesicular transport/endocytosis plays in the penetration of cells by Sindbis virus, and by extension, Alphaviruses. Many assays for virus infection published previously inferred that events related to virus infection had or had not occurred from observation of events that occur late in infection such as RNA or protein synthesis. In these cases penetration was not measured but rather the failure of an event occurring after penetration, genome translation and posttranslational processing as the indicator that penetration had not taken place. Our result differs from those experiments in that, while we are employing measurement of a late event (virus production in a plaque) to determine that infection has occurred, ours is a positive rather than a negative outcome and an indication that penetration must have taken place. In the case of a negative outcome where RNA or protein synthesis did not occur, many host factors of known and unknown host cell-mediated responses may have been involved in inhibition.

The data presented above suggest that interaction of virus with living cells at low temperature allows for conformational changes in the virion which place the virus beyond inactivation by antivirus serum. We found that the antiserum employed in these studies could bind to virus particles after they had undergone conformational changes leading to infection (Fig. 1). We also found that this antiserum could inactivate 86% of virus after attachment to cells at 5 °C for 15 min. We do not know if the 14% that escaped inactivation did so because they had penetrated cells in the 15 min time period or if this was just the limit of the ability of the antiserum to inactivate virus. If the values in Fig. 2 are corrected to remove the background 14% from each of the experiments we find that at 5 °C, 14.3% and 19.8% of attached virions escape antibody inactivation in 30 and 60 min. At 15 °C, 43% and 54% of attached virions escape antibody inactivation. The data presented in Fig. 3 indicate affirm that endocytosis does not occur at 5 °C and 15 °C. If endocytosis was simply slowed at these low temperatures and that slowing accounted for the approximately 50% of viruses attached infecting, a 50% of uptake of neutral red should have also been detected. Our results show that Sindbis virus can infect cells under conditions which block endocytosis.

We also examined the ability of Sindbis virus to mediate cell–cell fusion after transient exposure to acid pH at temperatures which block endocytosis. Cell fusion could be readily demonstrated at temperatures which permit endocytosis but could not be demonstrated at low temperature (Fig. 4). This may be related to a reduction in membrane fluidity at the low temperature (Moore et al., 1976). The finding that low pH-mediated fusion could not be demonstrated at 15 °C while penetration could take place at this low temperature (Figs. 2 and 4) separates the process of infection from membrane fusion and suggests that infection can take place in the absence of fusion.

The data presented in this paper add to a developing body of information suggesting that Alphaviruses can penetrate cells by a mechanism that does not require endocytosis, exposure to acidic pH or high concentrations of cholesterol in the target membrane (Koschinski et al., 2005; Paredes et al., 2004; Rietveld et al., 1999). The observation that events leading to infection can proceed at low temperature, conditions which prevent endocytosis and membrane fusion, support the notion that penetration occurs by production of a protein pore in the cell plasma membrane (Koschinski et al., 2005; Paredes et al.,

2004). Evidence for the existence of such Sindbis virus-induced pores produced in the plasma membranes of infected cells has existed for some time established by the observation that newly infected cells become leaky and ion-permeable (Ulug et al., 1984). This observation has been confirmed recently in experiments elaborating differences in pores formed as a result of penetration and pores formed as a result of membrane modification by virus proteins (Koschinski et al., 2005). Electron microscopy has also indicated the presence of a hollow connector developing between the virus and the cell surface during infection (Paredes et al., 2004). The early cytopathic effect in living cells exemplified by changes in ion permeability is in direct contrast to the fusion between virus and liposomes induced by low pH which is found to be non-leaky (Smit et al., 2002).

The difference in the structure of the envelope glycoproteins which make up the Type I and Type II "fusion" proteins suggest that while both classes of proteins are capable, under very specific conditions, of producing fusion of the virus membrane with a target membrane, they may function differently during the process of infection. Both types of proteins are capable of engaging cell membranes but Type I fusion proteins which are capable of inducing a fusion event may differ from Type II fusion proteins in that these proteins may, in concert with the virus receptor, also be capable of producing a protein pore in the host cell membrane. Infection of host cells by "Type II" proteins which are found in membrane containing viruses that must infect both vertebrates and invertebrates may require that they employ structural proteins in very different and unique ways to breach the barrier presented by these very different membranes. That the alternate, insect host, is poikilothermic and functions at ambient temperature may explain the ability of the Alphaviruses to infect cells at low temperatures.

## Methods

### *Cells and anti-Sindbis virus antibody*

Baby Hamster Kidney (BHK-21) cells were propagated in minimal essential medium containing Earl's salts (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 5% tryptose phosphate broth, and 2 mM glutamine as per standard protocol. Antibody against Sindbis viral proteins was produced in rabbits (Lampire, Pipersville, PA), and was the same as was used in previously published electron microscope studies (Paredes et al., 2004 and Fig. 1).

IgG was purified from serum using a HiTrap protein A column (Amersham, Uppsala, Sweden).

### *Virus growth and purification*

Sindbis virus was grown as described previously (Renz and Brown, 1976). Virus was twice purified by density gradient centrifugation on continuous potassium tartrate gradients. Purified virus was titered on BHK 21 cells and infectivity (particle to PFU ratio) was determined as described previously (Hernandez et al., 2003). The infectivity of the virus employed in these studies was 3 virus particles per infectious unit.

### *Electron microscopy*

Cell monolayer were fixed with paraformaldehyde followed by fixing with 3% glutaraldehyde in 0.1 M cacodylic acid buffer, pH 7.4 (Ladd Research Industries, Williston, VT) at 4 °C. The cells were washed three times with 0.1 M cacodylic acid, the cells were stained with 2% osmium tetroxide in cacodylic buffer for 1 h. Cells were then washed as before and embedded in 2% agarose. The agarose containing the cell sample was then prestained with 1% uranyl acetate (Polaron Instruments Inc, Hatfield, PA) overnight at 4 °C. The samples were washed and carried through ethanol dehydration. Infiltration was done using SPURR compound (LADD Research Industries). Blocks were then trimmed on an LKB NOVA Ultrotome (Leica Microsystems, Inc. Deerfield, IL). Ultrathin sections were stained with 5% uranyl acetate in distilled water for 60 min and in Reynolds lead citrate, pH 12 (Mallinkrodt Baker Inc. Paris, KY) for 4 min. The samples were examined at 80 kV in a JEOL JEM 100S transmission electron microscope.

### *Low-temperature infection initiation assay*

Equivalent monolayers of BHK cells were grown in 25 $cm^3$ cell culture flasks. Virus was attached to cell monolayers at 5 °C for 15 min while rocking, followed by incubation at 5 °C, 15 °C or 22 °C for 0 (control) 30 or 60 min. After removal of the inoculum, purified IgG was added to the virus–cell mixture at 5 °C for 60 min to inactivate virions that had not yet initiated infection. Cells were then immobilized with agarose in media and kept at 37 °C, 5% $CO_2$ for 36 h before plaques were counted.

### *Neutral red uptake assay and spectral analysis*

A neutral red uptake assay was employed to measure the ability of cells to take up macromolecules at different temperatures (Long et al., 2005; Weeks et al., 1987). To avoid crystal formation, neutral red solution was incubated in a 37 °C water bath overnight and filtered just before use. Cells were treated with 0.00025% neutral red in PBS-D and 3% serum for 30 min followed by a wash of the cell monolayer using ice cold PBS-D Cell associated neutral red was extracted using a 1:1 ratio of ethanol and acetic acid. Absorbance of the extract was measured at 550 nm using a Beckman DU 7500 spectrophotometer. All assays were done in triplicate. *p*-values were generated by the ANOVA procedure in SAS 8.0.

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