The Effect of pH on the Early Interaction of West Nile Virus with P388D1 Cells

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SUMMARY

The interaction between the flavivirus West Nile virus (WNV) and cells of the mouse macrophage-like cell line, P388D1, was assayed by transmission electron microscopy, by following the association of [³⁵S]methionine-labelled virus with cells, and by using a radiobinding assay with an ¹²⁵I-labelled F(ab')₂ fragment of a monoclonal antibody directed against the major viral envelope surface glycoprotein. Using electron microscopy, both fusion and endocytosis were observed at pH 6.4, but at pH 8.0 only endocytosis was observed. When ³⁵S-labelled WNV was bound to the P388D1 cell surface at 0 °C, less virus eluted on warming to 37 °C at mildly acidic than at alkaline or neutral pH values. The monoclonal antibody fragment had an increased affinity for cell surface viral E glycoprotein after prebound WNV was warmed at mildly acidic pH values. It is proposed that the warming of cell–virus mixtures at low pH results in fusion with a consequent reduction in elution of virus and an increase in the recognition of cell surface-expressed viral envelope glycoprotein by labelled antibody.

INTRODUCTION

The genome of an infecting enveloped animal virus is separated from the cytoplasm of a target cell by two lipid membranes, that of the virus and that of the cell. To penetrate these barriers, two pathways are generally considered (Dales, 1973; Longberg-Holm & Philipson, 1974).

First, viruses may fuse with the plasma membrane and thereby insert their genomes directly into the cytoplasm. Such fusion is best documented for Sendai virus and other paramyxoviruses (Choppin & Compans, 1975; Poste & Pasternak, 1978). The second route, which appears to be employed by several other enveloped viruses, is endocytosis of the particle into the cell, after which the viral genome is liberated into the cytoplasm (Dales, 1973; Longberg-Holm & Philipson, 1974). Recently, it has been convincingly shown that several enveloped viruses (White et al., 1980, 1983; Matlin et al., 1981, 1982; Yoshimura et al., 1982) can fuse efficiently with different target membranes at acidic, but not neutral pH and it has been proposed that fusion is involved in the infectious entry process of enveloped viruses, whereby viral genetic material is liberated into the cytosol either at the plasma membrane or from acidic endosomal compartments (Marsh, 1984).

We recently demonstrated the fusion activity of the flavivirus West Nile Virus (WNV) with artificial liposomal model membranes, which occurred in a pH-dependent manner (Gollins & Porterfield, 1986a). The present work investigates the situation with whole cells and presents biochemical and morphological evidence that WNV-induced membrane fusion is relevant to the viral entry process.

METHODS

Cells. Vero cells were grown in Dulbecco's modification of Eagle's MEM (Gibco) supplemented with 3% heat-inactivated (56 °C, 30 min) foetal calf serum (HIFCS; Gibco) and 0.3% bovine serum albumin (BSA; fraction V, t Present address: Department of Microbiology, Kansai Medical University, Moriguchi, Osaka 570, Japan.

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supplemented with 0.5% BSA and 15 mM-HEPES pH 7.6. During this procedure, brains were kept at 4 °C. The resulting suspension was clarified by centrifugation at 10,000 g for 1 h at 4 °C. The supernatant was then distributed into 0.1 ml aliquots and immediately frozen at −70 °C.

To obtain stocks of unlabelled, semi-purified WNV for electron microscopy and solid-phase radioimmunoassay, a modification of the method of Gollins & Porterfield (1985) was used. Briefly, slightly subconfluent Vero cells in 175 cm², 800 ml flasks (Nunc) were infected at an m.o.i. of 50 to 100, using a clarified infected suckling mouse brain suspension of WNV. Cells were incubated for 1 h at 37 °C, washed twice and then L-15 supplemented with 3% HIFCS and 0.3% BSA was added to each flask. Incubation was continued for 8 h, then the cells were washed three times and L-15, buffered with 10 mM-HEPES and 10 mM-EPPS pH 7-8 (Sigma), containing 0.3% BSA was added. Incubation was continued for a further 18 h and tissue culture supernatants were pooled and clarified at 10,000 g for 1 h in a Spinco SW28 rotor. The pellet was resuspended in 0.14 M-NaCl, 0.01 M-Tris-HCl, 0.2% BSA (pH 7.6) at 1/700 of the original volume of tissue culture supernatant and stored at −70 °C. This preparation contained 7 × 10^10 p.f.u. per ml (assayed in P388D1 cells). Purified 35S-labelled WNV was prepared essentially as described by Gollins & Porterfield (1984). The stock of virus contained 5.5 × 10^6 c.p.m./ml and 2 × 10^7 p.f.u./ml (assayed in P388D1 cells).

**Antibodies.** The monoclonal antibody F6/16A against WNV envelope E glycoprotein (Peiris et al., 1982) was purified from mouse ascitic fluid using Protein A-Sepharose (Pharmacia) by the method of Ey et al. (1978). The antibody was then dialysed against phosphate-buffered saline (PBS) and stored at −20 °C.

**Preparation of F(ab′)2 fragment of F6/16A.** The F(ab′)2 fragment of F6/16A was prepared by a modification of the method of Parham (1983). F6/16A at 5.4 mg/ml in 0.1 M-acetate buffer pH 4.1 was incubated with pepsin at 270 μg/ml of antibody solution for 3 h at 37 °C. The pepsin was then inactivated by the addition of 2 M-Trizma base and samples were analysed on 7.5% SDS-polyacrylamide gels with and without reduction as described by Laemmli (1970).

Pepsin-cleaved F6/16A was then put through a Protein A-Sepharose column to remove undigested IgG and fractionated on a Sephacryl S-300 superfine (Pharmacia) column in PBS/0.5 M-NaCl/10 mM-NaNO₃. Only the forward shoulder of the first protein peak was retained.

**Radioiodination of F(ab′)2 fragment of F6/16A.** The chloramine T method of Hunter & Greenwood (1962) was used as follows. Five μl of [125I]iodide (100 μCi/ml, carrier-free; Amersham), 15 μl of freshly made chloramine T at 0.25 mg/ml in 0.3 M-sodium phosphate buffer pH 7.3 and 2 μl of dimethyl sulfoxide were mixed together. The F(ab′)2 fragment (50 μl at 1 mg/ml) was then added and the mixture was incubated for 2 min with occasional mixing at room temperature. Ten μl of tyrosine (0.3 mg/ml in PBS) and 50 μl of HIFCS were then added to stop iodination. The mixture was loaded onto a 9 ml Sephadex G-25M (Pharmacia) column and the radiolabel in the excluded fraction was collected. In all cases the labelled antibodies were shown to be undenatured by binding to WNV antigens in competition with various known amounts of unlabelled antibody.

**Assay for the association of labelled virus with P388D1 cells.** The assay was a modified version of the method of Matlin et al. (1981). 35S-WNV was allowed to bind to monolayers of P388D1 cells plated on 35 mm diameter plastic Petri dishes (Falcon) for 120 min in a cold room on ice at pH 7.6. After this, free and loosely bound virus were removed by washing three times with ice-cold binding medium (L-15 supplemented with 0.5% BSA containing 15 mM-HEPES pH 7.6). The cells were then warmed for 1 or 5 min by addition of pre-warmed assay medium [L-15 supplemented with 0.5% BSA containing 10 mM-MES (Sigma) and 10 mM-HEPES] at various pH values in a 37 °C water-bath. After washing with ice-cold binding medium, cells were resuspended in 0.5 ml PBS and added to scintillation vials containing 0.1 ml 10% SDS in distilled water. Ten ml of Unisolve-E scintillation fluid (Koch-Light) was added, and radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer. Controls without virus or with radioactive virus bound on ice were also used. The background radioactivity in the absence of virus was always <5% of the positive controls, and was subtracted to give cell-associated radioactivity. All determinations of each value and virus-free backgrounds were performed in duplicate and the mean values were used for calculations.

**Assay for the amount of viral antigen on the surface of P388D1 cells.** Binding and internalization assays were all performed at a cell concentration of 2 × 10⁷ to 3 × 10⁷ P388D1 cells/ml in binding medium, pH 7.6. Cells were infected at an m.o.i. of 2.5 × 10² (p.f.u./cell) for 120 min on ice. The fusion assay was carried out in a water-bath at 37 °C in assay medium at various pH values.

The standard cell washing procedure involved adding 50 to 100 μl of an infected cell suspension to 1.5 ml of PBS supplemented with 0.5% BSA, pH 7.6 (PBS/BSA), pelleting the cells, and washing them twice more with 1.5 ml of PBS/BSA in 1.5 ml microfuge tubes (Sarstedt). For large quantities of cells, i.e. washing at 0 °C before a
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synchronized infection, cells were washed three times with 15 ml of PBS/BSA in 15 ml centrifuge tubes (Falcon). All cell pelleting and washing was carried out in a cold room.

For detecting cell surface-exposed viral antigen, the final cell pellet (10⁶ cells/sample) was resuspended with ¹²⁵I-labelled F(ab')₂, 2 × 10⁸ c.p.m. in 50 μl assay medium, pH 7·6 and allowed to react on ice for 60 min. Then cells were washed another three times, as above, and bound radioactivity was measured in a γ counter (Puckard Auto-Gamma Scintillation Spectrometer). The background radioactivity in the absence of virus was always <10% of the positive controls, and was subtracted to give the relative amount of surface-exposed antigen shown in the figures. All determinations of each value and virus-free backgrounds were performed in duplicate or triplicate and the mean values were used for calculations.

Antibody-binding solid-phase radioimmunoassays for detecting a pH-induced conformational change in the envelope E glycoprotein of WNV. The kinetics of the association and dissociation of F6/16A interacting with the envelope E glycoprotein of WNV was monitored by the method of Mason & Williams (1980). Semi-purified WNV was adsorbed overnight at 0 °C to the wells of a single lot of polystyrene Falcon micro-test plates. Empty wells were refilled with 5% BSA containing 15 mM-HEPES, 15 mM-MES, 0·12 M-NaCl (D-buffer with BSA: D/BSA), pH 7·6 and incubated for 2 h in the cold. For the association assay, the kinetics of the initial binding of the antibody were measured, when virions and iodinated antibody were incubated together for various intervals of time. D-buffer without antibody at various pH values was initially added to each well for 3 min at 37 °C. Wells were then washed and 2 × 10⁶ c.p.m. of ¹²⁵I-F6/16A in 50 μl of D/BSA, pH 7·6 was added for 1 min intervals up to 8 min in the cold. The wells were then washed with D/BSA and bound radioactivity was measured. Control experiments were carried out by omitting virus but otherwise being treated in the same way.

The kinetics of the dissociation of F6/16A was monitored as follows: the wells were treated with D-buffer at various pH values, as described above. The wells were next washed and a saturating amount of unlabelled F6/16A was added for 60 min in the cold. The wells were washed to remove free unlabelled antibody and then 2 × 10⁵ c.p.m. of ¹²⁵I-F6/16A in 50 μl of D/BSA, pH 7·6, was added for various time intervals up to 8 min in the cold. The wells were washed with cold D/BSA at the end of each time interval and bound radioactivity was measured. Control experiments were performed by omitting virus or by omitting unlabelled antibody but otherwise treated in the same way. The background radioactivity in the absence of virus was always <10% of specifically bound radioactivity (association kinetics) or <1% of specifically bound radioactivity in the absence of unlabelled antibody (dissociation kinetics). This value was subtracted to give all values shown in the figures. All determinations of each value and virus-free backgrounds were carried out in duplicate and the mean values were used for calculations.

Electron microscopy and immuno labelling of WNV with gold particles and attempts to detect direct fusion of virus with the plasma membrane at pH 6·4. These procedures were carried out essentially as described by Gollins & Porterfield (1985). WNV, in a volume of 30 μl PBS/BSA pH 7·6 (3·6 × 10² p.f.u./cell), was applied for 120 min at 0 °C to monolayers of P388D1 cells on 'Thermonox' tissue culture coverslips (Miles Laboratories) in wells of a 24-well Linbro plate. There were 1·7 × 10⁵ P388D1 cells/coverslip. These were washed in ice-cold PBS/BSA, pH 7·6 and used directly or allowed to warm up for various time intervals up to 5 min by addition of pre-warmed medium (pH 6·4 or 8·0, 37 °C) and flotation on a 37 °C water-bath. Cells were cooled by addition of ice-cold PBS/BSA and placed on ice. Fifty μl of F(ab')₂ of F6/16A (10 μg/ml, pH 7·6) was added for 60 min at 0 °C. The cells were washed in the cold, then gold-conjugated goat anti-mouse IgG (Miles Laboratories) was added for 60 min at 0 °C. The cells were washed in the cold, fixed and processed for transmission electron microscopy as described in Gollins & Porterfield (1985). Control experiments were done by omitting WNV but including both antibodies, or omitting both virus and F(ab')₂ fragment.

Quantitative electron microscopy. Single virus particles or aggregates of virus particles in the early stages of infection were counted under the electron microscope and the mean result from 100 cell profiles was taken for each final value.

Infectivity assay of the inoculated virus. The infectivity of the virus used was assayed by plaque titration in P388D1 cells, using the technique of Madrid & Porterfield (1969).

RESULTS

Electron microscopic evidence for fusion at low pH

The most direct and definitive method of determining whether or not fusion of a viral envelope with a plasma membrane has occurred is electron microscopy. When WNV was bound to P388D1 cells at pH 7·6 and 0 °C and warmed at 37 °C and pH 8·0, virus particles were seen both bound to the cell surface (Fig. 1 a), and entering the cell in the coated pits (Fig. 1 b) and coated vesicles (Fig. 1 c), as described by Gollins & Porterfield (1985). Some such virus particles could be immunologically labelled with gold particles (Fig. 1 a and b). There was no evidence of virus–cell fusion up to 5 min after warming.
Fig. 1. Entry of WNV into P388D1 cells by adsorptive endocytosis at pH 8.0. Cells were incubated for 2 h at 0 °C and pH 7.6, and infection was allowed to proceed synchronously by warming for 1 min at 37 °C at pH 8.0. The cells were incubated at 0 °C with F(ab')₂ of F6/16A and subsequently with gold-conjugated goat anti-mouse IgG before preparation for electron microscopy. (a) Single virus particles, labelled with several gold particles, attached to P388D1 cells. (b) Coated invagination of the plasma membrane endocytosing virus particles labelled with single gold particle at 1 min after warming. (c) Coated vesicle containing a single virus particle at 1 min after warming. Bar marker represents 100 nm.

If virion–cell complexes were warmed to 37 °C at pH 6.4 and fixed at 1 min after warm-up, fusion was observed (Fig. 2). Fusion occurred both at microvilli and at the cell surface but was never seen within the coated regions. Several stages of fusion could be observed. The distance between the viral and cellular membranes was 10 to 15 nm at the stage of attachment but was reduced to 5 nm or less as fusion progressed (Fig. 2a and b). The densely stained spherical nucleocapsid (diameter approx. 35 nm) then became clearly visible underlying the membrane (Fig. 2d) and overlaying E glycoprotein antigens, left on the cell surface, could be immunologically labelled with gold particles (Fig. 2c and d).

Quantitative electron microscopy on cell–virus interaction at pH 6.4 and 8.0

Table 1 demonstrates that after 1 min at pH 6.4, 33% of cell-associated virions were ‘fusing’ or ‘fused’ and only 12% were undergoing endocytosis. In contrast at pH 8.0, 62% of cell-associated virions were being endocytosed. No fusion was seen at pH 8.0. By 5 min after warming (Table 2), the number of cells with virions attached to their surface had significantly decreased. Neither at pH 6.4 or 8.0 were any ‘fusing’ virions observed at this time point. At pH 6.4 the ratio between ‘fusing and fused’ and ‘endocytosing’ virions was reversed (33:12 at 1 min and 20:38 at 5 min).

Association of ³⁵S-WNV with P388D1 cells: the effect of different pH values on prebound virions

To characterize the effect of pH on the association of virus with cells, the alteration of binding of ³⁵S-labelled WNV preadsorbed to P388D1 cells was measured. ³⁵S-WNV was allowed to bind with monolayers of P388D1 cells in a cold room on ice (i.e. at 0 °C to 4 °C), at which temperature no internalization occurred (Gollins & Porterfield, 1984). Fig. 3 shows that after incubation at 37 °C at pH 7.4 and 8.0 about 90% of the bound radioactivity eluted from the cells. However, less virus eluted from cells exposed to pH values below pH 7.4, with least elution occurring at pH 6.4 or below.

Binding of antibody to prebound virus: the effect of different pH values

The disappearance of envelope E glycoprotein of WNV prebound to P388D1 cells was monitored by the binding of ¹²⁵I-F(ab')₂ of F6/16A. The results in Fig. 4 show that at 1 min after warming at pH 7.6 and 8.0 the bound radioactivity on infected P388D1 cells was lower than that at time zero, presumably due to elution of virus (Fig. 3). However, when infected cells were exposed to pH values below pH 7.4, a progressive pH-dependent increase in bound radioactivity
Fig. 2. Fusion of WNV at the P388D1 plasma membrane at pH 6.4. Cells were incubated with virus for 2 h at 0 °C and pH 7.6 and then infection was allowed to proceed synchronously by warming for 15 s to 5 min at 37 °C and pH 6.4. The cells were then incubated at 0 °C with F(ab')2 of F6/16A and subsequently with gold-conjugated goat anti-mouse IgG before preparation for electron microscopy. (a) Viral particles labelled with several gold particles, attached to P388D1 cells at 15 s after warming. Arrow shows the close approach of the viral and plasma membranes. (b) A single virus particle labelled with gold particles fusing at the P388D1 plasma membrane at 15 s after warm-up. (c) A single virus particle labelled with a gold particle fused at the P388D1 plasma membrane at 1 min after warm-up. (d) A densely stained nucleocapsid (arrow) underlying the plasma membrane at 5 min after warm-up. Bar marker represents 100 nm.

Table 1. Quantitative electron microscopy on WNV interaction with P388D1 cells at 1 min after warm-up*

<table>
<thead>
<tr>
<th>Virion-bearing cells</th>
<th>pH 6.4</th>
<th>pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-associated virion number (per cell profile)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>55/100</td>
<td>55%</td>
</tr>
<tr>
<td></td>
<td>55/100</td>
<td>55%</td>
</tr>
<tr>
<td></td>
<td>194/55</td>
<td>3-5</td>
</tr>
<tr>
<td></td>
<td>177/56</td>
<td>3-2</td>
</tr>
<tr>
<td>Fusing + fused</td>
<td>41 + 23/194</td>
<td>33%</td>
</tr>
<tr>
<td></td>
<td>0/177</td>
<td>0%</td>
</tr>
<tr>
<td>Endocytosing</td>
<td>23/194</td>
<td>12%</td>
</tr>
<tr>
<td></td>
<td>110/177</td>
<td>62%</td>
</tr>
<tr>
<td>Attached (i.e. non-fusing and non-endocytosing)</td>
<td>107/194</td>
<td>55%</td>
</tr>
<tr>
<td></td>
<td>67/177</td>
<td>38%</td>
</tr>
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</table>

* Quantitative electron microscopy was performed as described in Methods. The classification of the interaction between virions and P388D1 cells was determined as described in the legends to Fig. 1 and 2.

was seen at 1 min after warm-up to levels greater than at time zero with a maximum occurring at pH 6.4 or below (Fig. 4, closed circles). In other words, there was an increase in available virus antigen (see Discussion). There was a 3.5-fold difference between radioactivity bound after treatment at pH 8.0 and that at pH 6.4.

When the incubation was carried out for up to 5 min at 37 °C (Fig. 4, open circles), the relative amount of exposed antigen at all pH values examined was lower than that seen after 1 min
Fig. 3. Association of $^{35}$S-WNV with P388D1 cells: the effect of different pH values on prebound virions. $^{35}$S-WNV was allowed to bind to cells for 120 min at 0 °C in binding medium, pH 7.6. The cells were warmed to 37 °C for 1 min (●) or 5 min (○) by adding prewarmed media at various pH values and analysed for cell-associated radioactivity. Four-thousand c.p.m. was initially bound to cells before warming.

Fig. 4. Binding of F6/16A to prebound virus: effect of pH. The relative amount of surface-exposed viral antigen at various pH values was assayed using $^{125}$I-F(ab')$_2$ of F6/16A as described in Methods. The assay was for 1 min (●) or 5 min (○) at 37 °C. After the initial washing at 4 °C, a total of 2550 c.p.m. of $^{125}$I-F(ab')$_2$ of F6/16A was bound/10$^6$ cells. The results are expressed as the percentage of the amount of virus bound initially.

Table 2. Quantitative electron microscopy on WNV interaction with P388D1 cells at 5 min after warm-up*

<table>
<thead>
<tr>
<th>Virion-bearing cells</th>
<th>pH 6.4</th>
<th>pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25/100</td>
<td>19/100</td>
</tr>
<tr>
<td>Cell-associated virion number (per cell profile)</td>
<td>25%</td>
<td>19%</td>
</tr>
<tr>
<td>Fusing + fused</td>
<td>80/25</td>
<td>68/19</td>
</tr>
<tr>
<td>0 + 16/80</td>
<td>20%</td>
<td>0%</td>
</tr>
<tr>
<td>Endocytosing</td>
<td>30/80</td>
<td>48/68</td>
</tr>
<tr>
<td>38%</td>
<td>71%</td>
<td></td>
</tr>
<tr>
<td>Attached (i.e. non-fusing and non-endocytosing)</td>
<td>34/80</td>
<td>20/68</td>
</tr>
<tr>
<td>42%</td>
<td>29%</td>
<td></td>
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</tbody>
</table>

* The classification of the interaction between virions and P388D1 cells was determined as described in the legends to Fig. 1 and 2.
incubation. This was surprising since in Fig. 3 more virus was cell-associated at 5 min than at 1 min, but may reflect internalization of E antigens into the cell.

**Binding of antibody to prebound virus: the effect of increasing m.o.i.**

The amount of antibody binding to virus was dependent on the input multiplicities. Using the results in Fig. 5 an m.o.i. of $2.5 \times 10^2$ was chosen for the experiments described below.

**Binding of antibody to prebound virus: the effect of time**

Time course experiments showed that at pH 6.4 the increase in recognizable antigen occurred very rapidly and was essentially complete by 75 s after warming to 37 °C (Fig. 6). 85% of maximal antigen increase was induced within a 15 s warming period in pH 6.4 medium. At pH 8.0, only a progressive decrease of bound radioactivity was observed.

**Binding of antibody to prebound virus: the effect of temperature**

At pH 6.4, an increase in binding of F6/16A antibody was observed over a wide temperature range, with a maximal increase in binding occurring at 37 °C (Fig. 7). In contrast, at pH 8.0 bound radioactivity decreased at all temperatures.

**Kinetics of the association and dissociation of F6/16A using WNV particles pretreated at different pH values**

The kinetics of the association of F6/16A with WNV particles bound to plastic plates were monitored from the initial reaction rate measured by incubating $^{125}$I-F6/16A with WNV pretreated with various pH buffers. In Fig. 8(a), the time course of the initial reaction is shown for F6/16A with WNV under conditions of antigen excess. For F6/16A the reaction had the characteristics of single-hit kinetics. The association kinetics at various pH values were identical.

The dissociation kinetics were determined by incubating WNV, prebound to plastic plates, with saturating amount of unlabelled antibody, washing the plates, and then observing the association of trace amounts of newly added labelled antibody with viral antigens. Using this indirect assay, the association of labelled F6/16A was thus a measurement of the dissociation kinetics of unlabelled F6/16A. Fig. 8(b) shows that the kinetics of the dissociation of F6/16A followed the kinetics of a multi-order reaction. Dissociation kinetics were not affected by the various pH values used.

Saturation experiments (not shown) showed that WNV particles maximally bound a similar number of antibody molecules, irrespective of the pH of pretreatment.
DISCUSSION

This paper presents evidence based principally on electron microscopy that WNV (family Flaviviridae) can fuse with the plasma membrane of the mouse macrophage-like cell line P388D1, with a maximum at mildly acidic pH values (Fig. 2).

To follow WNV binding to the cell surface and subsequent internalization, it proved necessary to use high numbers of viral particles per cell. The viral preparations used routinely had a particle : infectivity ratio of between 200 and 600. Nevertheless, it is probable that the majority of virus particles in such preparations are potentially infectious (Gollins & Porterfield, 1985). The high particle : infectivity ratio probably reflects the inefficiency of WNV binding to the cells in the absence of antibody. Binding can be significantly improved by adding subneutralizing anti-WNV antibody concentrations (Gollins & Porterfield, 1984).

Less prebound virus eluted at pH 6.4 than at pH 8.0 (Fig. 3). This increased affinity of WNV particles at lowered pH values could be due to the fusion of virus particles with the cell surface or intermediate stages in the fusion process seen by electron microscopy (Fig. 2).

The greater amount of 35S-labelled virus attached at 5 min than at 1 min at pH 7.0 and below must be because eluted virus is re-attaching to cells (Fig. 3). The reason for this is unclear. It has been shown by Gollins & Porterfield (1986b) that when viral infectivity is measured in experiments similar to this, there is a large (90%) reduction in infectivity between pH 7.4 and
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Fig. 8. The kinetics of the association and dissociation of F6/16A interacting with the envelope E glycoprotein of WNV. WNV preadsorbed to wells of micro-test plates was incubated for 3 min at 37 °C in buffers at various pH values. (a) For the association assay, the kinetics of the initial binding of the antibody were monitored as described in Methods for up to 8 min at 0 °C and pH 7.6. (b) For the dissociation assay, the association kinetics of 125I-F6/16A was followed instead, after preincubation of WNV with saturating amounts of unlabelled antibody for up to 3 h at 18 °C and pH 7.6. ●, pH 7.6; ○, pH 7.4; ■, pH 6.8; □, pH 6.4; △, pH 6.0; △, pH 5.4; ▽, pH 5.0.

6.4. However, the reductions in infectivity occurring with 1 min or 5 min treatment at pH 6.4 are approximately equal, meaning that the re-attaching virus in Fig. 3 is non-infectious (not shown).

The relative amount of surface-exposed viral antigen (the major E envelope glycoprotein), was investigated using the monoclonal antibody F6/16A (Fig. 4 to 7). When cells with prebound WNV particles on their surface were warmed for brief periods below pH 7.0, an increase in recognizable antigen occurred to levels greater than at time zero (Fig. 4). This meant that as well as reducing virus particle elution (Fig. 3), treatment at pH 6.4 and 37 °C also made E glycoprotein molecules more accessible to antibody. One possibility is that this could have been due to lateral diffusion of E glycoprotein molecules in the cell membrane, following fusion events similar to those seen under the electron microscope (Fig. 2). In support of this, the pH-dependent increase in E antigen recognized was quite similar to the pH dependence of fusion between WNV and artificial liposomes (Gollins & Porterfield, 1986a). The time dependence (Fig. 6) and temperature dependence (Fig. 7) of this process were also similar.

Alternatively, the increase in detectable viral antigen at lowered pH could have resulted from a conformational change in the E glycoprotein itself, as shown for influenza virus (Yewdell et al., 1983) and Sindbis virus (Edwards et al., 1983), resulting in an increased physical availability of recognizable antigenic epitopes for F6/16A. However, solid-phase radioimmunoassays (Fig. 8a and b) show no change in the affinity of F6/16A for virus pretreated at subneutral pH, and thus argue against a conformational change occurring in the E glycoprotein molecule. It should be borne in mind, however, that antigenic or physical changes can occur when virus particles are bound to plastic (Nestorowicz et al., 1985; N. J. Dimmock, personal communication), so that this conclusion cannot be made with absolute certainty.

The pH dependence of WNV-induced fusion is quite similar to that for haemagglutination of erythrocytes by WNV, which usually has a pH optimum of 6.5 (Porterfield & Rowe, 1960). The possibility thus arises that haemagglutination is actually due to a fusogenic interaction between
erythrocytes and virus particles. Similarly with alphaviruses, haemagglutination, haemolysis and erythrocyte fusion all occur at a similar pH optimum (5.8) (Väänänen & Kääriäinen, 1980), which indicates they are possibly all manifestations of the same phenomenon.

However, the optimum pH for haemolysis by several flaviviruses, including WNV, has recently been found to be 5.4, with no haemolysis detectable above pH 6.0 (Cammack & Gould, 1985). It is possible that WNV particles do fuse with erythrocyte membranes at pH 6.4, so that one virus particle can cross-link two erythrocytes, although further changes occurring at pH 5.4 are necessary for leakage of haemoglobin.

It has been proposed that an acid-catalysed fusion event is important in the escape of WNV genomes from prelysosomal vacuoles into the cytosol (Gollins & Porterfield, 1985). The present results support this proposition in that fusion was seen electron microscopically between viral and cell membranes at mildly acidic pH (Fig. 2). At pH 6.4, both direct fusion and endocytosis were seen, whereas only endocytosis was seen at pH 8.0. Thus the proportion of virus fusing increased at the lower pH. The results in the $^{35}$S- and $^{125}$I-binding studies can both be readily interpreted in terms of virus–cell plasma membrane fusion.

Direct fusion at low pH with the cell plasma membrane does not seem to be the mechanism by which WNV infects cells since the virus fused at low pH with P388D1, L929 and PS clone D cell lines was non-infectious (Gollins & Porterfield, 1986b). It would appear that a low pH environment must be encountered in an intracellular endosome in order for infectious uncoating to take place and the reasons for this have been discussed (Gollins & Porterfield, 1986b).

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REFERENCES


Entry of flavivirus


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