Flavivirus Infection Enhancement in Macrophages: an Electron Microscopic Study of Viral Cellular Entry

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SUMMARY

The mode of entry of West Nile virus (WNV) into the macrophage-like cell line P388D1 was investigated at the electron microscopical level using synchronized infections. The presence of the antiviral monoclonal antibody F6/16A at a concentration that enhanced viral attachment to P388D1 cells ninefold made no difference to the entry pathway of WNV. In both the absence and presence of F6/16A the initial uptake of single viral particles was mediated by coated pits, and started within 30 s of warming the cells to 37 °C. Viral particles later appeared in fully or partially coated vesicles and later in uncoated prelysosomal endocytic vacuoles before degradation in lysosomes. However, aggregates of viral particles (five or more virus particles in cross-section), appeared to be phagocytosed whole by cells in a process which involved aggregates being engulfed by extensions of the plasma membrane. This process exhibited a slower time course than the uptake of single viral particles, becoming prominent 15 to 30 min after warming the cells to 37 °C. The involvement of a prelysosomal vacuolar compartment in the entry process was shown by a failure to stain for acid phosphatase. This compartment could be specifically loaded with viral particles when viral internalization occurred at 20 °C in the presence of 50 mM-ammonium chloride.

INTRODUCTION

Infection of cells of the monocyte-macrophage lineage or macrophage-like cell lines with flaviviruses in the presence of subneutralizing antiviral antibody can cause an increase in viral replication compared to controls where infection is carried out in the absence of antibody (Halstead & O'Rourke, 1977; Peiris & Porterfield, 1979; Schlesinger & Brandriss, 1981; Brandt et al., 1982). This has been shown (Gollins & Porterfield, 1984) to be due to an increase in numbers of virus particles binding to the cell surface and to a higher specific infectivity of antibody-coated cell surface-bound virus particles due to a more efficient internalization process.

For an increasing number of physiologically important macromolecules, including some enveloped viruses, it has been shown that entry into cells is mediated by receptor-mediated endocytosis (Goldstein et al., 1979; Brown et al., 1983; Pastan & Willingham, 1983; Marsh, 1984). Ligand-receptor complexes firstly cluster in clathrin-coated pits on the cell surface. These are then internalized to form coated vesicles which then lose their coat and fuse with other intracellular vesicles to form endosomes. From this compartment, the ligand either escapes to the cytoplasm or is delivered to the Golgi system, or undergoes degradation after delivery to secondary lysosomes.

No morphological studies have, as yet, been done on virus entry using accessory receptors such as the Fc receptor (FcR) or complement receptor (CR3) (Cardosa et al., 1983). Thus, the present study was carried out in order to determine morphologically what the entry process of West Nile virus (WNV) into P388D1 cells appeared to be at the electron microscopical level, and whether the viral entry mechanism differed in the absence and presence of antiviral antibody.
**METHODS**

**Cells.** Vero cells, L929 cells and P388D1 cells were all maintained as described previously (Gollins & Porterfield, 1984).

**Virus.** Unlabelled virus was prepared by infecting slightly subconfluent Vero cells in 75 cm² flasks (Falcon) with 50 p.f.u./cell, using a clarified infected suckling mouse brain suspension of WNV. The inoculum was left on the cells for 1 h at 37 °C. The cells were washed twice and then Dulbecco's MEM, buffered with 10 mM-HEPES and 10 mM-EPPS [4-(2-hydroxyethyl)-1-piperazinepropanesulphonic acid] (pH 7-6), containing 0-2% bovine serum albumin (BSA) was added to each flask. Infection was allowed to proceed for 32 h and tissue culture supernatants were pooled and clarified at 10000 g for 1 h. Virus was then pelleted by centrifugation at 50000 g for 3 h in a Spinco SW28 rotor. The pellet was resuspended in 0-13 m-NaCl, 0-02 m-Tris-HCl, 0-2% BSA (pH 7-4) at 1/200 of the original volume of tissue culture supernatant and stored at -70 °C. This preparation contained 4 \times 10^{11} virus particles per ml (as judged by latex particle counts) and had a particle to p.f.u. ratio of approximately 500 (assayed in L929 cells).

Radioactively labelled 35S-WNV was prepared as described previously (Gollins & Porterfield, 1984). The stock of virus used contained approximately 10^{10} virus particles/ml, contained 5 \times 10^{6} c.p.m./ml and there were approximately 500 virus particles/p.f.u. (assayed in L929 cells).

**Antibody.** The IgG2a monoclonal antibody F6/16A against WNV (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.

**Electron microscopy.** Non-radioactive WNV, in a volume of 20 μl (5 \times 10^{4} virus particles/cell), was bound for 2 h at 0 °C to monolayers of P388D1 cells on coverslips placed in wells of a 24-well Linbro plate. The coverslips were 8 x 8 mm square, manually cut from 'Thermanox' tissue culture coverslips (Miles Laboratories) and there were 1-2 x 10^{3} P388D1 cells/coverslip. These were then washed twice in ice-cold binding medium (L15 medium, 0-2% BSA, 15 mM-HEPES pH 7-4) and either fixed directly, or pre-warmed binding medium was added at 37 °C, the Linbro plates were floated on a 37 °C water-bath to synchronize internalization of virus and cells were fixed at various times after warm-up. Cells were fixed for 30 min in ice-cold 2-5% glutaraldehyde and post-fixed in 1% osmium tetroxide (both in 0-1 M-cacodylate buffer pH 7-3). The cells were then washed in distilled water, block-stained with 2% aqueous uranyl acetate solution, dehydrated in graded ethanol solutions and embedded. The monolayers were sectioned still attached to the coverslips and the sections were finally stained for 10 min with lead citrate (Reynolds, 1963), before examination under the electron microscope.

**Quantitative electron microscopy.** This was carried out by counting numbers of single virus particles or aggregates of virus particles bound or internalized per cell profile under the electron microscope, and averaging the results from 300 cell profiles for each final value.

**Staining for acid phosphatase.** This was performed essentially as described by Barka & Anderson (1962). Monolayers of P388D1 cells were fixed for 30 min with ice-cold 2-5% glutaraldehyde in 0-1 M-cacodylate buffer (pH 7-3), and then washed overnight in cacodylate buffer. Sections were then washed for 20 min in two changes of Tris–maleate buffer (pH 5-2), and incubated for 1 h 15 min at room temperature in the Barka and Anderson modification of Gomori's medium, containing 8 mM-sodium β-glycerophosphate. They were then washed for 60 min with two changes of Tris–maleate buffer (pH 5-2), post-fixed for 30 min in 1% osmium tetroxide in 0-1 M-cacodylate buffer (pH 7-3), dehydrated, embedded and sectioned for electron microscopy. Sections were examined directly without additional staining. Negative controls either lacked the substrate (sodium β-glycerophosphate) or included 10 mM-sodium fluoride in washing and incubation solutions as an inhibitor.

**Immune labelling of WNV particles with gold particles and attempts to detect direct fusion of viral particles with the plasma membrane at pH 7-4.** WNV was bound to P388D1 cell monolayers at a concentration of 5 \times 10^{4} particles per cell in the presence of 10 μg/ml F6/16A for 2 h on ice. The cells were then washed in the cold and used directly, or allowed to warm up for 1 min intervals up to 5 min by addition of pre-warmed binding medium (37 °C) and flotation on a 37 °C water-bath. The cells were then cooled by addition of ice-cold medium and placed on ice. Goat anti-mouse IgG, conjugated to 10 nm diameter gold particles (Miles Laboratories), was then added for 1 h at 0 °C. The cells were then washed in the cold, fixed and processed for electron microscopy. Control experiments were carried out by omitting WNV but including F6/16A, or omitting both WNV and F6/16A.

**Binding assay for 35S-labelled WNV attachment to cells.** Monolayers of P388D1 cells grown on 8 x 8 mm coverslips were washed twice in binding medium and 35S-labelled WNV (178 virus particles/cell; 11786 c.p.m./coverslip) with or without additional unlabelled WNV (5 \times 10^{4} virus particles/cell) was added to each coverslip in a total volume of 20 μl. The monoclonal antibody F6/16A was included at various concentrations and binding was allowed to proceed for 2 h at 0 °C. Coverslips were then washed three times in cold binding medium, cells were solubilized in 1% NP40 in distilled water, and bound c.p.m. determined.

**Infectivity assays of bound virus.** The infectivity of bound virus was determined in monolayers of P388D1 cells parallel to those in which bound c.p.m. was measured. Monolayers were washed in the cold, warm growth medium
Fig. 1. Binding of WNV to P388D1 cells at two different virus to cell ratios in the presence of various concentrations of F6/16A. Binding of 35S-labelled WNV for 2 h at 0 °C, to confluent monolayers of P388D1 cells (178 particles/cell, 11786 c.p.m./coverslip) was performed in the absence (●) or presence (○) of additional unlabelled WNV (5 × 10⁴ particles/cell in the presence of various concentrations of the antiviral antibody F6/16A. Bound c.p.m. was assayed by solubilizing the cells in 1% NP40 as described in Methods.

Table 1. **Injectivity of WNV in P388D1 cells at two different particle to cell ratios in the presence or absence of 'enhancing' antibody**

<table>
<thead>
<tr>
<th>Titre of infectious virus produced in 2-day tissue culture supernatants (p.f.u./ml × 10⁻³)</th>
<th>3sS-WNV alone (178 virus particles added per cell)</th>
<th>3sS-WNV (178 virus particles per cell) + unlabelled WNV (5 × 10⁴ virus particles added per cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>58</td>
<td>612</td>
</tr>
<tr>
<td></td>
<td>(No F6/16A)</td>
<td>(No F6/16A)</td>
</tr>
<tr>
<td></td>
<td>(+ 1 μg/ml F6/16A)</td>
<td>(+ 10 μg/ml F6/16A)</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>693</td>
</tr>
<tr>
<td></td>
<td>612</td>
<td>725</td>
</tr>
</tbody>
</table>

* Performed as described in Methods using the antibody concentrations noted in the Table.

was added, and productive infection allowed to proceed for 2 days at 37 °C. The yield of infectious virus produced in tissue culture supernatants was then assayed by plaque titration in L929 cells, using the technique of Madrid & Porterfield (1969).

**RESULTS**

**Establishment of conditions for enhanced binding of WNV to P388D1 cells**

In order to be able to visualize virus particles bound to the cell surface in the absence of antibody, and to follow their subsequent cellular entry pathway using transmission electron microscopy, it was found necessary to use a high multiplicity of virus particles per cell (5 × 10⁴ per cell). To ensure that antibody was present at a concentration that would enhance binding of viral particles to the cell surface, binding assays using 35S-labelled WNV were performed at 0 °C under exactly the same conditions that were used in the electron microscopical study. Binding of
Table 2. Quantitative electron microscopy on the attachment of single particles and aggregates of WNV to P388D1 cell monolayers*

<table>
<thead>
<tr>
<th>Number attached to plasma membrane per cell profile</th>
<th>No F6/16A</th>
<th>+ F6/16A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single viral particles: 2 h binding at 0 °C</td>
<td>1.4</td>
<td>11.5</td>
</tr>
<tr>
<td>Aggregates of virus particles (two or more): 2 h binding at 0 °C</td>
<td>0.065</td>
<td>0.6</td>
</tr>
<tr>
<td>Single viral particles: 2 h binding at 0 °C then 1 h at 37 °C</td>
<td>0.29</td>
<td>1.4</td>
</tr>
<tr>
<td>Aggregates of virus particles (two or more): 2 h binding at 0 °C then 1 h at 37 °C</td>
<td>0.034</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* WNV particles were bound to coverslip cultures of P388D1 cells at 5 x 10^4 particles/cell in the absence or presence of 10 μg/ml F6/16A. They were then washed in the cold and either fixed directly or allowed to warm up to 37 °C for 1 h before fixation and preparation for electron microscopy. Quantitative electron microscopy was performed as described in Methods and each figure represents the average of 300 cell profiles.

35S-WNV was examined in the absence or presence of a 280-fold excess of unlabelled WNV (Fig. 1). It was seen that antibody could enhance the binding of virus as the concentration was increased, and approximately tenfold more antibody was needed to enhance binding of 35S-WNV in the presence of the higher multiplicity of virus. Using these results, an antibody concentration of 10 μg/ml was chosen for electron microscopic studies using the higher viral multiplicity (5 x 10^4 virus particles/cell).

The relevance of the binding enhancement to infectivity enhancement was established by determining the infectivity of bound virus, as described in Methods (Table 1). One μg/ml of F6/16A produced a 31-fold increase in viral yield using 35S-WNV alone. In the presence of unlabelled WNV, little enhancement was seen with 10 μg/ml F6/16A despite viral binding being enhanced ninefold (Fig. 1), probably because the maximum number of cells had been productively infected in the absence of antibody.

Quantitative electron microscopy on the binding of single or aggregated virus particles to P388D1 cells at 0 °C and clearance of pre-bound virus at 37 °C

Quantitative electron microscopy demonstrated that approximately eightfold more single virus particles and ninefold more aggregates of virus particles bound to the cell surface in the presence than in the absence of antiviral antibody (Table 2).

Approximately 5% of particles attached to the cell membrane were present as aggregates (i.e. two or more viral particles) both in the absence and presence of antibody. This result showed that at the virus and antibody concentrations used in this electron microscopical study, antibody enhanced the binding of single and aggregated particles to the cell surface but did not itself induce aggregation of virus particles.

When suspensions of WNV particles from the stock used for the electron microscopical study were allowed to bind to parlodion- and carbon-coated grids and then stained with 2% aqueous uranyl acetate, it was also seen that approximately 5% of particles consisted of aggregates, i.e. two or more virus particles bound together (not shown). When virus was held at pH 7.4 at 0 °C in the absence of cells and antibody, the proportion of aggregated particles did not increase with time (up to 6 h examined). This demonstrated that the aggregates were present only as a result of the viral preparation procedure and that viral particles did not undergo spontaneous aggregation at neutral pH.

After warming of the cells to 37 °C for 1 h, the vast majority of single virus particles had disappeared from the cell surface both in the presence and absence of antiviral antibody. Radioactivity studies showed that in the presence of antibody more than 95% of virus particles remained associated with cells, though in the absence of antibody more than 25% of virus particles eluted from the cell surface on warming to 37 °C. Most cell-associated particles were internalized both in the absence and presence of antibody (see below). In the presence of antibody, aggregates were cleared efficiently from the cell surface, although in the absence of
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Fig. 2. Binding of WNV particles to the P388D1 cell membrane at 0 °C in the absence and presence of antibody. WNV particles were bound for 2 h at 0 °C to P388D1 cells and then the cells were washed in the cold and prepared for electron microscopy, without warming up, as described in Methods. (a) Single viral particle bound in the absence of antibody. (b, c) Single particles, and (d) an aggregate of WNV particles bound in the presence of 10 μg/ml F6/16A. Note the absence of antibody in (a) and the presence of antibody (arrowed), as fine strands or aggregated material in (b), (c) and (d). Bar marker represents 100 nm.

Antibody over half the aggregates that had initially bound were still attached to the cell surface at the end of the warm-up period. This might have been because multiple FcR interactions in the presence of antibody promoted phagocytosis of larger aggregates of viral particles.

Appearance of virus particles after binding to the plasma membrane at 0 °C for 2 h

After viral particles had been bound for 2 h at 0 °C to P388D1 cell monolayers, particles were distributed fairly evenly over the plasma membrane. Virus particles had a diameter of approximately 40 nm and no evidence of entry into cells via coated pits was seen at this time. In the absence of antibody virus particles appeared smooth-surfaced (Fig. 2a). In the presence of F6/16A, antibody could be seen attached to the surface of some virus particles as a coating of fine strands and aggregated material that had sometimes become partly detached from the virus surface during fixing and staining (Fig. 2b, c, d).

Location of virus particles during the first 5 min after a synchronized infection at 37 °C

No difference was seen temporally or morphologically in the entry pathway for single virus particles that had bound to cells in the absence of antibody and those which had bound in the presence of antibody. For this reason, when describing the entry pathway below, no distinction is made between entry in the absence and presence of antiviral antibody.

Within 30 s of warm-up, virus particles appeared in invaginations of the plasma membrane that bore an electron-dense thickened coat (Fig. 3a, b). Slightly later, at 1 min after warm-up, most viral particles were located in these invaginations or in small vesicles of approximate diameter 100 to 150 nm that were also coated (Fig. 3c) and had probably resulted from the coated invaginations budding into the cytoplasm. These structures appeared very similar to the coated pits and coated vesicles reported in the entry of several viruses into cells (Marsh, 1984).
Fig. 3. Location of WNV particles 30 s to 3 min after warming to 37 °C. WNV particles were allowed to infect P388D1 cells synchronously at 37 °C. They were then fixed and prepared for electron microscopy 30 s to 3 min after warm-up, as described in Methods. (a, b) Coated invaginations of the plasma membrane endocytosing virus particles at 30 s after warm-up. (c) Coated vesicle containing a single virus particle at 1 min after warm-up. (d, e) Single virus particles in partially coated vesicles at 2 min after warm-up (coated region, large arrows; virus particles, small arrows). (f, g) Single virus particles in uncoated vesicles 3 min after warming to 37 °C. (b, c, d, f) No F6/16A; (a, e, g) F6/16A at 10 μg/ml. Bar marker represents 100 nm.

Approximately 1 to 2 min after warm-up, virus particles were seen in partially coated vesicles that appeared to have resulted from fusion of virus-containing coated vesicles with other small cytoplasmic vesicles (Fig. 3d, e). It is likely that in the images seen, the vesicles were undergoing uncoating because at 3 min after warm-up viral particles were seen in small uncoated vesicles (Fig. 3f, g). This indicated that coated vesicles were short-lived intracellularly and lost their coat within 1 or 2 min after formation.

Between 3 and 5 min after warming to 37 °C, viral particles appeared in large, uncoated, electron-lucent vesicles more than 700 nm in diameter, which had presumably arisen from fusion of smaller uncoated vesicles either with each other, or with a large vesicular compartment already present (Fig. 4a, b). These vesicles were shown to be prelysosomal using the stain for acid phosphatase (see below).
Fig. 4. Location of WNV particles 3 to 5 min after warming to 37 °C. WNV particles were allowed to infect P388D1 cells synchronously at 37 °C. The cells were then fixed and processed for electron microscopy 3 to 5 min after warm-up as described in Methods. (a, b) Large, electron-lucent vesicles more than 700 nm in diameter containing several virus particles. (a) No F6/16; (b) F6/16A at 10 μg/ml. Bar marker represents 100 nm.
Fig. 5. Labelling of WNV particles with gold-conjugated antibody. (a) WNV particles were allowed to bind to the P388D1 cell plasma membrane for 2 h at 0 °C in the presence of 10 μg/ml F6/16A. The cells were then washed in the cold and labelling was performed for 1 h at 0 °C with gold-conjugated goat anti-mouse IgG. They were then washed, fixed directly, and prepared for electron microscopy as described in Methods. The arrows point to gold particles bound to an aggregate of WNV particles. In (b) and (c) the cells were allowed to warm up for 30 s before cooling on ice and gold labelling. Single viral particles are seen labelled with single gold particles in the initial stages of entry into cells in coated pits. Bar marker represents 100 nm.

Labelling of viral particles with gold-conjugated antibody

The identity of WNV particles was confirmed by indirect labelling in the cold with gold-conjugated goat anti-mouse IgG, as described in Methods. Fig. 5(a) shows an aggregate of viral particles coated with F6/16A and specifically labelled with gold-conjugated goat anti-mouse IgG (gold particles arrowed).

The background labelling of the plasma membrane due to FcRs on the cell surface was negligible. This might have been because the input of 5 × 10^4 virus particles/cell largely absorbed most of the F6/16A added, leaving relatively few single antibody molecules free to bind to FcRs. This explanation received support from control experiments with F6/16A but without WNV. In this case substantial labelling of the plasma membrane was seen. Gold-conjugated goat anti-mouse IgG was shown to have a low affinity for mouse FcRs because controls without virus or F6/16A showed little labelling of the plasma membrane.

Attempts to detect direct fusion of WNV with the plasma membrane of P388D1 cells at pH 7·4 and 37 °C

Because of the very low background labelling of the plasma membrane, it was possible to look for direct fusion of WNV with the plasma membrane at pH 7·4 as a mechanism for entry of WNV. If fusion occurred directly between the cell and viral membranes, then viral antigens
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Fig. 6. Location of viral particles 15 to 30 min after warming to 37 °C. WNV particles were allowed to infect P388D1 cells synchronously at 37 °C in the presence of 10 μg/ml F6/16A. The cells were then fixed and prepared for electron microscopy after 15 to 30 min as described in Methods. (a) Two virus particles (arrowed) in a lysosome 30 min after warming to 37 °C. (b, c, d) Entry pathway for aggregates: (b) an aggregate of virus particles being phagocytosed by a P388D1 cell; (c) an aggregate of viral particles in the process of phagocytosis completely enclosed by membrane; (d) an aggregate of viral particles in an intracellular vesicle completely surrounded by membrane. Bar marker represents 100 nm.

would be left on the cell surface after fusion had occurred. When such viral antigens were looked for, as described in Methods, no specific labelling of the plasma membrane was seen up to 5 min after warming to 37 °C at pH 7-4. This ruled out direct fusion with the plasma membrane at neutral pH as a major mechanism by which viral RNA gained access to the cytosol. However, within the first minute after warm-up, virus particles entering the cells via coated pits could be labelled in the same experiments (Fig. 5b, c).

Location of virus particles 15 to 30 min after warming to 37 °C: lysosomal location of single virus particles and phagocytic uptake of aggregates of virus particles

It is known from degradation studies using [35S]methionine-labelled WNV (Gollins & Porterfield, 1984) and [3H]uridine-labelled WNV (S. W. Gollins & J. S. Porterfield, unpublished) that from 10 min onwards in a synchronized infection, WNV particles start to reach the lysosomal compartment where they are rapidly degraded to TCA-soluble material.
Thus, at 30 to 45 min after warm-up, many intracellular viral particles will be in the lysosomal compartment. Morphologically the lysosomal vacuoles reached at 30 to 45 min after warm-up were little different from large prelysosomal endocytic vacuoles except that their lumen often contained electron-opaque granular material (Fig. 6a) or membranous material.

The rate of uptake of aggregates containing more than five viral particles in cross-section was lower than that of single viral particles and they appeared to undergo a form of phagocytosis. Whole aggregates were initially surrounded by plasma membrane (Fig. 6b). They were then completely enclosed by plasma membrane (Fig. 6c) and subsequently appeared enclosed in discrete uncoated vesicles within the cytoplasm (Fig. 6d).

The internalization of aggregates took appreciably longer than that of single viral particles, and images such as those in Fig. 6(b, c, d) were seen mainly 15 to 30 min after warming to 37 °C.

**Demonstration of a prelysosomal compartment in the entry of WNV into P388D1 cells and loading of this compartment with viral particles**

When a synchronized infection of P388D1 cells was allowed to proceed for 5 min at 37 °C and the cells were then fixed and stained for acid phosphatase as described in Methods, it was found that many of the uncoated vesicles containing viral particles (Fig. 4), could not be stained and were thus non-lysosomal (not shown).

At 20 °C, in a variety of cell types, including macrophages, material can enter prelysosomal endocytic compartments from the plasma membrane, but the passage to secondary lysosomes is blocked (Helenius et al., 1983). It is also known that weak bases such as ammonium chloride and chloroquine can raise intraendosomal and intralysosomal pH (Ohkuma & Poole, 1978). In the case of Semliki Forest virus this pH increase can inhibit an acid-triggered fusion reaction between endosomal and viral membranes and thus inhibit penetration of viral RNA to the cytosol (Helenius et al., 1982).

In order to attempt to demonstrate a prelysosomal endocytic compartment in WNV internalization more clearly, synchronized internalization was carried out for 1 h 40 min at 20 °C in the presence of 50 mM-ammonium chloride.

Under these conditions many more virus-containing vesicles were seen than at any time point in experiments where synchronized internalization was carried out at 37 °C in the absence of ammonium chloride. Moreover, of the vesicles that did contain virus particles, many were completely full of particles. It proved possible, in the same cell section, to be able to stain lysosomes for acid phosphatase and to show that almost all the vacuoles loaded with viral particles could not be stained and were thus prelysosomal (Fig. 7). In the presence of 10 mM-sodium fluoride or in the absence of substrate (β-glycerophosphate), no specific labelling of lysosomes occurred. This showed that the stain for acid phosphatase used was specific for lysosomes only.

The two treatments appeared to be quantitatively additive in that when synchronized internalization occurred at 20 °C in the absence of ammonium chloride, or at 37 °C in the presence of 50 mM-ammonium chloride, increased numbers of virus particles were seen in non-lysosomal intracellular vacuoles compared to controls where internalization was carried out at 37 °C in the absence of ammonium chloride. In neither case, however, were the prelysosomal vacuoles loaded with virus particles so dramatically as when both treatments were used together.
Similar results were obtained whether virus was bound to cells in the absence or presence of F6/16A, though on quantitatively different scales according to the amount of virus initially bound to the plasma membrane.

**DISCUSSION**

This paper describes the entry pathway into cells adopted by the flavivirus WNV in the absence or presence of 'enhancing' antiviral antibody, using transmission electron microscopy. In order to visualize the viral binding to the cell surface and subsequent internalization in the absence of antibody, it proved necessary to use high numbers of viral particles per cell. When such high viral inputs are used, the virus : antibody ratio is increased and it was necessary to verify that antibody was present in concentrations that enhanced viral binding to the cell surface (Fig. 1). With the higher viral input in Fig. 1 (and that which was used for electron microscopical studies), cells appeared to be maximally productively infected in the absence of antibody and an enhancement of infectivity could not be demonstrated (Table 1), even though viral binding was enhanced ninefold at the antibody concentration used (Fig. 1).

We are aware that a major problem in the interpretation of electron microscopical investigations of viral entry processes is that in most viral preparations that have been used for electron microscopical studies, the particle : p.f.u. ratio is more than 10 (and often more than 100) (Dimmock, 1982). Thus, the possibility exists that when viral entry processes are visualized by electron microscopy the entry pathways seen for the majority of particles may not be relevant to the actual productive infection of cells that is accomplished by a relatively small minority of infectious virus particles, conceivably via a different entry mechanism. This problem would appear to be particularly marked in the case of WNV because our viral preparations routinely have a particle : p.f.u. ratio of between 200 and 600.

However, such particle to p.f.u. ratios are usually expressed on the basis of the ability of virus particles in the whole suspension to produce plaques in monolayers of susceptible cells. We have found that a major factor limiting viral infectivity in the case of WNV is that under the usual conditions of infection, less than 5% of viral particles in a suspension will actually bind and enter a variety of cell types, regardless of whether the cells are permissive to WNV infection or not (S. W. Gollins & J. S. Porterfield, unpublished). Against this it could be argued that the particles that enter cells truly represent infectious particles in the viral population and that the remainder are non-infectious simply because they are altered in some way so that they cannot bind to, or penetrate cells.

However, the flavivirus enhancement system offers good evidence that the latter is not the case. When specific infectivity of viral particles bound to the P388D1 cell surface by subneutralizing polyclonal rabbit anti-WNV antibody was examined (Gollins & Porterfield, 1984), the particle : p.f.u. ratio was 155 times higher than that for WNV particles in the viral suspension as a whole, when assayed on susceptible (L929) cells. Thus, it is probable that in this electron microscopical study a reasonable percentage of virus particles in the preparations used are actually infectious, despite the particle to p.f.u. ratio of 500 when assayed in L929 cells. Because morphologically only one entry pathway for single virions was seen in this study it is likely that this is the pathway adopted by infectious virions in the absence and presence of antibody.

The entry route visualized for single virus particles under the electron microscope did not differ in the absence or presence of antibody and appeared to be via the receptor-mediated endocytic entry route described for many other viruses (Marsh, 1984). The partially coated vesicles seen 1 to 2 min after warm-up (Fig. 3d, e) were very similar to those recently described in the entry pathways of poliovirus type 1 (Zeichhardt et al., 1985) and frog virus 3 (Braunwald et al., 1985). It will be interesting to verify whether the ability to internalize aggregates of five or more particles as seen in cross-section by what appeared to be a phagocytic mechanism (Fig. 6b, c, d) is due to the macrophage-like nature of P388D1 cells, or whether it also occurs in cell types unlike macrophages.

It was found that the large electron-lucent vacuoles seen 3 to 5 min after the start of entry of single virus particles, and the initial phagocytic vacuoles containing aggregates, could not be
stained with acid phosphatase and were therefore prelysosomal. When a synchronized internalization of WNV was carried out either at 20 °C or in the presence of 50 mM-ammonium chloride, an increase in the numbers of electron-lucent vesicles containing virus particles was seen, appearing as in Fig. 4. Because they could not be stained for acid phosphatase they were prelysosomal. When internalization of virus particles was carried out both at 20 °C and in the presence of 50 mM-ammonium chloride, the effect was dramatic. A large increase in the numbers of virus-containing vesicles was seen and nearly all of these were completely filled with virus particles (Fig. 7). Under these conditions almost all virus-containing vesicles were shown to be prelysosomal (Fig. 7).

These results have two implications. Firstly, they confirm that at 20 °C in P388D1 cells, as in other cell types (Helenius et al., 1983), entry into cells can take place but transport to lysosomes from prelysosomal endocytic compartments is blocked. Secondly, it is known that weak bases can inhibit flavivirus infection in P388D1 cells (Gollins & Porterfield, 1984; Brandriss & Schlesinger, 1984) and it has been shown here that 50 mM-ammonium chloride alone can inhibit penetration of WNV particles from prelysosomal vacuoles. This is probably due to the raising of intravacuolar pH in acidic vacuoles (Ohkuma & Poole, 1978), which in turn suggests an acid-triggered fusion reaction between WNV and prelysosomal vacuolar membrane as the mechanism for entry of WNV RNA to the cytosol, as with Semliki Forest virus (Helenius et al., 1982).

If, as seems likely, an acid-triggered fusion reaction is involved in WNV entry, it has proved extremely difficult to visualize under the electron microscope. This might be because the capsid–RNA complex either migrates rapidly away from the cytoplasmic side of the prelysosomal vesicle after fusion, or rapidly loses its conformation and capsid-like appearance. Finally, because the endocytic vesicular compartments that occur in the entry pathway of WNV into macrophages are prelysosomal (Fig. 7), it seems reasonable to refer to them as 'endosomes', in keeping with the terminology used by others (Helenius et al., 1983).

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