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An Unusually High Divalent Cation Requirement for Attachment of West Nile Virus to Primary Chick Embryo Cells (40046)

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The *in vitro* assay of West Nile virus (WNV) has met with varying degrees of success in the hands of different investigators who have employed a wide variety of cell systems and experimental conditions. Henderson and Taylor (1) were unable to demonstrate either CPE or plaque formation in chick embryo (CE) cell cultures. However, Buckley (2) reported that WNV produced both CPE and plaques in CE cultures but the *in vitro* titers were considerably lower than those achieved in experimental animals. Similar results were obtained by Westaway (3) using PS cells (a stable line of porcine kidney cells). A wide variety of primary cell culture systems (1, 4-9) and continuous cell lines (2, 10, 11) have been employed to demonstrate the capacity of WNV to cause CPE and/or plaque formation. Most of these studies were more or less screening tests of a qualitative nature designed to demonstrate the ability of WNV to cause CPE or plaques rather than a systematic examination of conditions for a sensitive *in vitro* assay for WNV. The variations in CPE and plaque formation are understandable in view of the diverse experimental procedures employed. Little has been done to elucidate the conditions requisite for an accurate *in vitro* assay of WNV, although in a preliminary report Eylar and Wisseman (12) reported that the sensitivity of the WNV plaque assay was influenced by the pH of the adsorption diluent. This communication reports an unusually high divalent cation requirement for maximal attachment of WNV to primary CE cells.

Materials and methods. Virus. The Egypt 101 strain of WNV, which had been passed serially in mouse brains through an undetermined number of passages, was employed in this investigation. Stock virus, as 20% suckling mouse brain suspensions in 50% normal, heat-inactivated rabbit serum-saline with 250 units/ml penicillin and 125 μg/ml streptomycin sulfate, was stored in sealed glass ampoules at −70°.

Cell cultures. Primary CE cell cultures were prepared by the method of Dulbecco (13) with minor modifications. Trypsinized cells were suspended in 5% heat-inactivated calf serum-yeast extract medium (14) with penicillin and streptomycin added to a final concentration of 250 units/ml and 125 μg/ml respectively and, dispensed in 5 ml amounts into 60 × 30 mm plaque bottles (15).

In vitro assay. The growth medium was removed and the monolayers rinsed with Hanks' balanced salts solution (BSS). Unless otherwise specified, virus dilutions were prepared in borate-KCl buffer at pH 8.3-8.5 so that there were approximately 1000 PFU/ml. Four to six replicate bottles containing 0.1 ml inoculum were incubated at 26° with intermittent rocking for 90 min. In experiments where adsorption had to be interrupted prior to completion, adsorption was halted by addition of 3-4 ml Hanks' BSS, the virus-BSS mixture removed and the wash procedure repeated twice. Monolayers were overlayed with a medium consisting of 2% calf serum, 0.25% lactalbumin hydrolysate, BSS without phenol red and 0.8% noble agar. Sodium bicarbonate was added to a final pH of 7.3-7.4. Bottles were inverted and incubated at 37° for three days when a second overlay consisting of 2% noble agar in BSS with 1:20,000 neutral red was added. Plaques were counted after overnight incubation at 37°.

Results. Influence of divalent cation concentration on plaque count. During preliminary experiments in which diverse diluents were employed, it became apparent that components within the diluent markedly influenced the final plaque count. Since cations and especially divalent cations, are known to influence attachment of certain

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viruses to host cells (16–19), the possibility of a cation requirement in the WNV-CE cell culture system was investigated. Accordingly, plaque assays were performed with mixtures in which $MgSO_4$ at various concentrations was added to a constant amount of WNV in borate-KCl diluent at pH 8.3–8.5. The results of these experiments (Fig. 1) clearly show that the $MgSO_4$ concentration played an important role in the productive adsorption of WNV to CE cells. Little adsorption occurred when the $MgSO_4$ concentration was less than 1×10^{-4} *M*, but with increasing $MgSO_4$ concentration plaque counts increased to a maximum at approximately 0.1 *M* $MgSO_4$. Similar results were obtained when $MgCl_2$, $BaCl_2$ or $CaCl_2$ were employed. To determine if these results were influenced by anions and to determine the influence of monovalent cations upon WNV adsorption, similar concentrations of Na^+, K^+, Mg^{++}, Cl^- and $SO_4^=$ in various combinations were employed in the adsorption medium. Of these, only Mg^{2+} increased plaque counts. Several other cations were also tested (viz., Fe^{2+}, Al^{3+}, Sn^{2+}, Mn^{2+}) but, of all of the cations utilized, only Mg^{2+}, Ba^{2+} and Ca^{2+} significantly increased the plaque counts.

The influence of Mg^{2+} concentration on the rate of WNV adsorption to CE cultures. The time course of adsorption of WNV to CE cell cultures, at four different Mg^{2+} concentrations, was studied. Adsorption was allowed to continue for predetermined time intervals when the infected cultures were washed with BSS and overlaid with the agar medium.

Figure 2 shows the results of this study. Adsorption was most rapid when 0.1 *M* Mg^{2+} was incorporated in the virus inoculum, and adsorption was essentially complete within 60 min. If the Mg^{2+} concentration was suboptimal (less than 0.1 *M*), adsorption was much slower and maximal adsorption was not achieved in the 300-min time period studied.

Discussion. An unusually high divalent cation concentration, with activity restricted to certain members of the cation group, was found for optimal attachment of WNV to, and maximal assay sensitivity in, CE cells. A 200-fold increase in plaque count

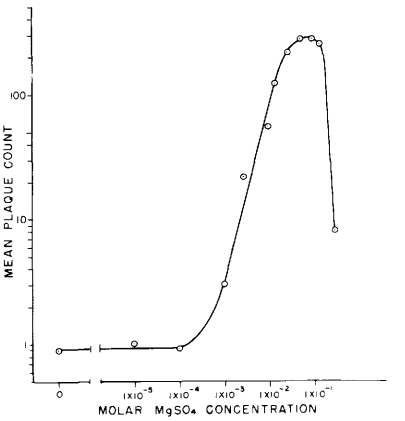

FIG. 1. The relationship between magnesium ion concentrations and relative WNV plaque count. A constant virus inoculum was mixed with different concentrations of magnesium sulfate in borate buffer and assayed in primary chick embryo cells.

from the same virus input occurred when the final Mg^{2+} concentration in the adsorption medium was raised from 1×10^{-4} *M* to 1×10^{-1} *M*. At approximately 1×10^{-3} *M* Mg^{2+} or Ca^{2+} (the approximate concentration of these ions in Hanks' BSS) there is minimal enhancement of WNV adsorption to CE cultures. This perhaps explains in part the varied results obtained when BSS or BSS containing diluents were employed in preliminary experiments. With BSS diluents, slight alterations in the cation concentration would result in exaggerated changes in the plaque count.

Influence of divalent cations on adsorption of viruses to host cells has been reported occasionally. For example, attachment of specific phages to *E. coli* (16), *Pasteurella* (17) or *Salmonella* (18) has a divalent cation requirement as does Japanese encephalitis virus to hamster kidney cells (19), and dengue virus to Rhesus monkey kidney cells (20). However, cation requirements are not necessarily uniform within a broad virus group. While added divalent cations did enhance the attachment of coxsackie A9 virus to human amnion cells (21), divalent cations were not essential for optimal attachment of poliovirus to HeLa cells (22). Attachment of Venezuelan equine encephalomyelitis virus to McCoy cells was optimal with 0.10–0.15 *M* NaCl while low levels of Ca^{2+} or Mg^{2+} inhibited

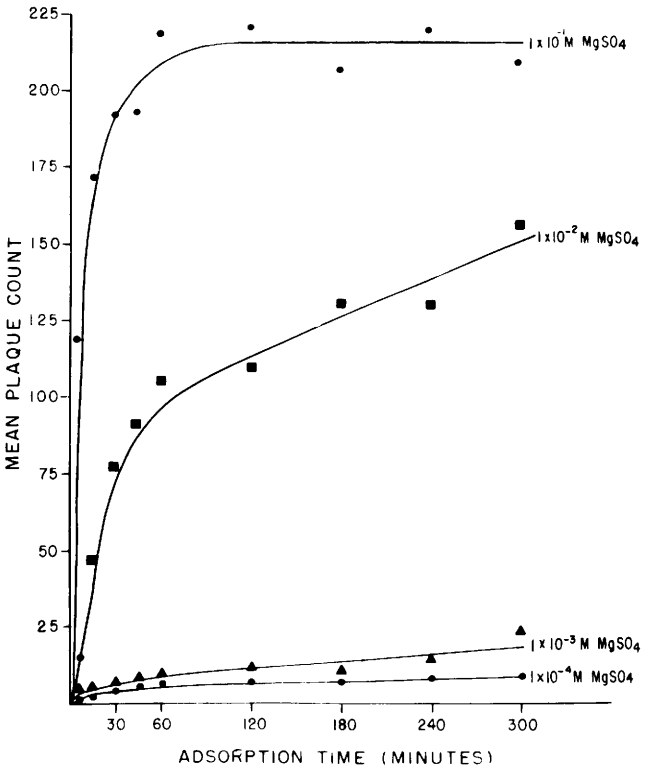

FIG. 2. Adsorption of WNV to chick embryo monolayer cultures from 0.1 ml inoculum at 26° at various $MgSO_4$ concentrations. Stock virus was added to borate-KCl buffer with 1×10^{-1} *M* $MgSO_4$ (●——●), 1×10^{-2} *M* $MgSO_4$ (■——■), 1×10^{-3} *M* $MgSO_4$ (▲——▲) and 1×10^{-4} *M* $MgSO_4$ (●——●). At the indicated time intervals replicate cultures were removed, adsorption halted by repeated washes with BSS and overlayed with agar.

virus attachment (23). Thus, cation enhancement of attachment of a virus to host cell most likely reflects a specific host cell-virus requirement rather than a general characteristic of the virus *per se*.

The enhanced WNV attachment to CE cells could not be attributed to (1) stabilization of WNV by Mg^{2+}, as has been demonstrated with the enteroviruses, because equivalent WNV stability was observed with or without 0.1 *M* Mg^{2+} (data not shown), (2) pH stability because the stability of WNV at pH 7.0 to pH 8.5 remained relatively constant (data not shown), (3) Mg^{2+} reversal of borate interactions with the carbohydrate component of virus or host cell glycoprotein (24) because a similar Mg^{2+} enhancement could be demonstrated when tris buffer was substituted for borate buffer, (4) increased virus yield as a result of promoting migration of viral nucleocapsids to the cell membranes (25) because increased Mg^{2+} levels were required for the virus attachment process only. In addition it was found that increased levels of Mg^{2+} enhanced WNV attachment to BHK-21/15 cells but not to the extent observed in the CE cell-WNV system.

Thus, the divalent cation requirement for optimal attachment of WNV to CE cells appears to be unrelated to pH stabilization, Mg^{2+} stabilization, buffer effects, virus replication and assembly or to the source of the host cell. It is more likely that Mg^{2+}, and to a lesser degree Ca^{2+} and Ba^{2+} serve as a mechanism by which the negatively charged virus and host cell can come into intimate contact to initiate the adsorption and penetration process.

Summary. An unusually high divalent cation requirement for maximal plaque count and maximal adsorption rate was found in the WNV-CE cell system. An increase in Mg^{2+} concentration from 1×10^{-3} to $1 \times$

10^{-1} *M* resulted in a 200-fold increase in average plaque count. When 1×10^{-1} *M* Mg^{2+} was incorporated in the virus inoculum, WNV attachment was essentially complete in 50–60 min. If 1×10^{-2} *M* or less Mg^{2+} was included in the adsorption diluent, maximal attachment was not complete within 300 min and only a fraction of the original virus content could be detected by plaque assay.

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