Journal of NeuroVirology, 8: 100–110, 2002
 ISSN 1355–0284/02 $12.00+.00
DOI: 10.1080/13550280290049561

Mutation in loop I of VP1 of Theiler's virus delays viral RNA release into cells and enhances antibody-mediated neutralization: A mechanism for the failure of persistence by the mutant virus

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The DA strain of Theiler's murine encephalomyelitis viruses (TMEV) causes a central nervous system (CNS) demyelinating disease with viral persistence despite the presence of high serum anti-TMEV antibody titers. The DA virus mutant, T81D, was created to have a mutation at position 81 in loop I of VP1, close to the putative virus receptor binding site. T81D showed slower replication *in vitro* and *in vivo*. T81D-infected mice developed anti-TMEV antibody responses with no virus persistence. We tested whether the differences between the viruses were due to alteration in virus-cell interactions, or in the resistance to neutralization by anti-TMEV antibody. Using radiolabeled viruses, we found no difference in binding to permissive cell lines between the mutant and wild-type viruses. In a semipermissive cell line, DA virus bound more efficiently than T81D. During the uncoating step, both viruses decapsidated without the production of stable intermediates and 80% of viruses were eluted or decapsidated after 45 minutes. At the final step of uncoating, however, T81D showed a slower rate of RNA release than DA virus into cells using a photoinactivation assay. Anti-TMEV monoclonal and polyclonal antibodies neutralized T81D virus more efficiently than DA virus in suspension. Further, these anti-TMEV antibodies were able to neutralize viruses that had already attached to cells but not internalized (postadsorption neutralization [PAN]). However, DA virus showed significant resistance to PAN after cells were incubated at 37°C compared with T81D-infected cells. The development of resistance to PAN appeared to correlate with the rate of RNA release from virions into cells. In T81D virus infection, the slow RNA release and high susceptibility to neutralization by antibodies would result in a failure to establish virus persistence *in vivo*. Conversely, rapid RNA release and resistance to neutralization could favor virus persistence in DA virus infection. *Journal of NeuroVirology* (2002) **8,** 100–110.

Keywords: Theiler's virus; attachment; mutation; uncoating

Introduction

The family *Picornaviridae* is subdivided into nine genera: *Enterovirus* (poliovirus and coxsackievirus), *Rhinovirus* (rhinovirus), *Cardiovirus* (Encephalomyocarditis (EMC) virus), *Aphthovirus* (foot-and-mouth disease virus), *Hepatovirus* (hepatitis A virus), *Parechovirus*, *Erbovirus*, *Kobuvirus*, and *Teschovirus* (King *et al*, 1999, 2000, reviewed in Minor *et al*, 1995). The entry process of picornaviruses involves extensive virus-host interactions and can be divided into several initial parts or stages. These are binding, uncoating, and penetration (Rueckert, 1996). First, picornaviruses bind to their specific cellular receptors in the plasma membrane. Receptors for a variety of picornaviruses have been identified. Poliovirus uses the poliovirus receptor (PVR) and the major group of rhinoviruses use intercellular

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We thank Jana Blackett, Thomas S Cannon, Li-Qing Kuang, MD, and Isack ZM Igenge for technical assistance. We thank Ms Kathleen Borick for preparation of the manuscript. This work was supported by National Institutes of Health, NS 34497.

Received 24 August 2001; revised 10 December 2001; accepted 14 December 2001.

adhesion molecule-1 (ICAM-1) (Greve *et al*, 1989; Roivainen *et al*, 1994). Both receptors are members of the immunoglobulin superfamily. The receptor for the minor group of rhinoviruses is the low-density lipoprotein (LDL) receptor, whereas Coxsackievirus A9 and foot-and-mouth disease virus use integrins for cell entry (Hofer *et al*, 1994; Mason *et al*, 1994; Roivainen *et al*, 1994).

Theiler's murine encephalomyelitis viruses (TMEV) are a species in the genus *Cardiovirus* and are divided into two groups. The GDVII subgroup (GDVII and FA) is highly neurovirulent whereas the other subgroup, TO (DA, TO, WW, and BeAn), is less neurovirulent and causes a persistent infection with chronic inflammatory demyelination in the central nervous system (CNS) (Tsunoda and Fujinami, 1999). Although another immunoglobulin superfamily molecule, vascular cell adhesion molecule-1 (VCAM-1), is known to act as a receptor for the D variant of EMC virus that belongs to the genus *Cardiovirus* (Huber, 1994), the receptor for TMEV has not been identified. However, P0 of peripheral nerve may be a receptor outside the CNS (Libbey *et al*, 2001). Nevertheless, binding studies with TMEV to various cell lines have been performed to learn more about virus-receptor interactions (Rubio *et al*, 1990; Fotiadis *et al*, 1991; McCright and Fujinami, 1997). It was found that GDVII and BeAn strains of TMEV share a common receptor (Fotiadis *et al*, 1991). In other studies we and others found that there was no clear correlation between binding of virus to cells and susceptibility to infection (Rubio *et al*, 1990; McCright and Fujinami, 1997).

It has been proposed that the receptor for picornaviruses has a dual function: binding of the virus particle to the cell and subsequent release of the viral genome (Zajac and Crowell, 1969). The release of the viral genome into the cytoplasm upon binding consists of a series of steps that are not well defined or characterized. The virus needs to uncoat (at least partially) in order to release its genome into the cytoplasm of the host cell. The uncoating steps of poliovirus, rhinovirus, and coxsackievirus have been somewhat defined. Temperature and pH can influence these steps. The virion undergoes a series of stepwise uncoating reactions and intermediates of uncoating have been isolated. Upon binding to the receptor, the virus particle releases VP4 (Crowell and Philipson, 1971), the capsid swells, and the amino termini of VP1 protrudes toward the virion's surface (Fricks and Hogle, 1990). The resultant altered particle (A particle) is more lipophilic than is whole virus. This irreversible process is called the eclipse phase.

The final uncoating step, the release of RNA through the membrane, is still obscure and there is some controversy about where the dissociation of viral capsid with release of RNA takes place and whether through internalized endosomes or at the plasma membrane. In poliovirus infection, it has been proposed that the amino terminus of VP1 interacts with hydrophobic regions of the plasma membrane in cell entry (Lonberg-Holm *et al*, 1976; Fricks and Hogle, 1990; Belnap *et al*, 2000). Virus has been shown to cause ion-permeable channels in the lipid bilayer that could provide a pathway for transport of RNA across the plasma membrane (Tosteson and Chow, 1997). It is generally assumed, however, that the majority of A particle conversion occurs at the plasma membrane due to the fact that large numbers of A particles are sloughed off, eluted, and can be isolated from the supernatants of infected cells. Furthermore, the eclipse can occur in the presence of soluble receptors (Greve *et al*, 1989; Kaplan *et al*, 1990).

Intermediate forms of polioviruses, coxsackieviruses and rhinoviruses (*Enterovirus* and *Rhinovirus*) are generated during the uncoating step, which starts with the conversion to the largely non-infectious A particles (Crowell and Philipson, 1971; Curry *et al*, 1996). The intermediates of uncoated particles can be identified based upon their altered sedimentation profiles. Whole particles sediment at 160S, whereas A particles sediment at 135S. Empty capsids sediment at 80S and the pentamers or smaller subunits sediment at 15S or smaller. In contrast, EMC virus, which belongs to the genus *Cardiovirus* as does TMEV, is believed to uncoat without the generation of stable intermediates (Hall and Rueckert, 1971; McClintock *et al*, 1980). Foot-and-mouth disease virus (*Aphthovirus*) is also believed to uncoat without the generation of stable intermediates (Baxt and Bachrach, 1980).

The virus entry process can be monitored further by using neutral red (NR)-labeled virus. In poliovirus infection, release of the viral RNA into the cell occurs subsequent to the conformational change. At this stage, virions that have matured in the presence of photoreactive dyes become light insensitive. Thus, the loss of light sensitivity of dye-containing virus particles has been correlated with RNA release in picornavirus infection, such as in poliovirus, rhinovirus, and echovirus infections (Eggers and Waidner, 1970; Kirkegaard, 1990; Rosenwirth *et al*, 1995). Mutations in VP1 of poliovirus have been demonstrated to affect release of viral RNA but not the rate of binding to cells or the rate of subsequent receptor-dependent conformational changes (Kirkegaard, 1990).

A mutant DA strain of TMEV, T81D, with a threonine to aspartate substitution at position 81 in loop I of VP1 exhibited a large plaque phenotype, but had a slower replication cycle *in vitro* than that of wild-type DA virus (pDA virus) (McCright *et al*, 1999). Mice infected either with pDA or with T81D virus developed significant anti-TMEV antibody titers 1 week after infection (McCright *et al*, 1999). During the acute phase of TMEV infection (1 week postinfection), T81D virus-infected mice showed higher antibody titers than pDA virus-infected mice. However, during

the chronic phase, pDA virus-infected mice developed significantly higher antibody titers than those seen in T81D virus infection. Despite the high serum anti-TMEV antibody titer, pDA virus can persistently infect the spinal cord of the mice, leading to an inflammatory demyelinating disease, which is used as an animal model for multiple sclerosis (Tsunoda and Fujinami, 1999). In contrast, T81D virus showed a mild acute disease with infection of neurons and perivascular cells, mutant virus was eradicated and mice were not persistently infected.

Anti-virus antibody has been demonstrated to neutralize the virus by several different mechanisms (Outlaw and Dimmock, 1991; Vrijsen *et al*, 1993). Antibody can neutralize viruses in suspension by impeding cell attachment (standard neutralization [STAN]). In addition, antibody can act on virus that has already attached to cells but not internalized (postadsorption neutralization [PAN]). Thus, anti-virus antibody can inhibit postbinding events, such as internalization and uncoating of virus, even though virus has attached to the host cell (Vrijsen *et al*, 1993; Virgin *et al*, 1994).

In this paper, we first compared different aspects of viral entry between pDA and T81D viruses. We found no difference in binding between the viruses to permissive cell lines. However, T81D virus showed a lower rate of RNA release than pDA virus. Next, we compared the abilities of anti-TMEV monoclonal antibodies and polyclonal antisera to effect PAN and STAN between pDA and T81D viruses. We found that anti-TMEV antibodies neutralized T81D virus more efficiently than pDA in suspension (STAN). In addition, pDA virus showed significant resistance to PAN as early as 10 minutes after incubation at 37°C compared with T81D virus.

Results

Similar binding between pDA and T81D viruses to permissive cell lines To determine whether there was a difference in binding between wild-type pDA and the mutant T81D viruses, studies using radiolabeled virus and various cell lines were performed. No difference in binding was found using two permissive cell lines: BHK-21 and L929 (Figure 1). Binding of both viruses to BHK-21 cells increased over time, with maximum binding (30%) occurring at 30 min (Figure 1A). Binding to L929 cells reached levels of 20% after 45 min (Figure 1B). A competitive inhibition experiment with BHK-21 cells indicated that both viruses were equally inhibited with excess cold pDA virus (Figure 2). Although low levels of binding were seen with the CNS-derived cell lines, Neuro-2a and primary astrocytes, no difference was seen in the binding characteristics (Figure 3). In contrast, there was a change in binding between the two viruses to BSC-l cells, a semipermissive cell line (McCright and Fujinami, 1997) (Figure 4). The pDA virus bound BSC-l cells with up to 45% binding at 30 min whereas the T81D mutant reached levels of 23% after 45 min.

pDA and T81D viruses uncoat without production of stable intermediates, and have similar elution/decapsidation rates The T81D virus has a mutation in an area that can interact with a cellular receptor (McCright *et al*, 1999). Because the receptor could function not only in virus binding but also at a downstream event such as uncoating, we compared uncoating profiles between pDA and T81D viruses. Uncoating was examined by allowing radiolabeled virus to bind to cells in the cold, shifting the reaction to 37°C, followed by separation of whole

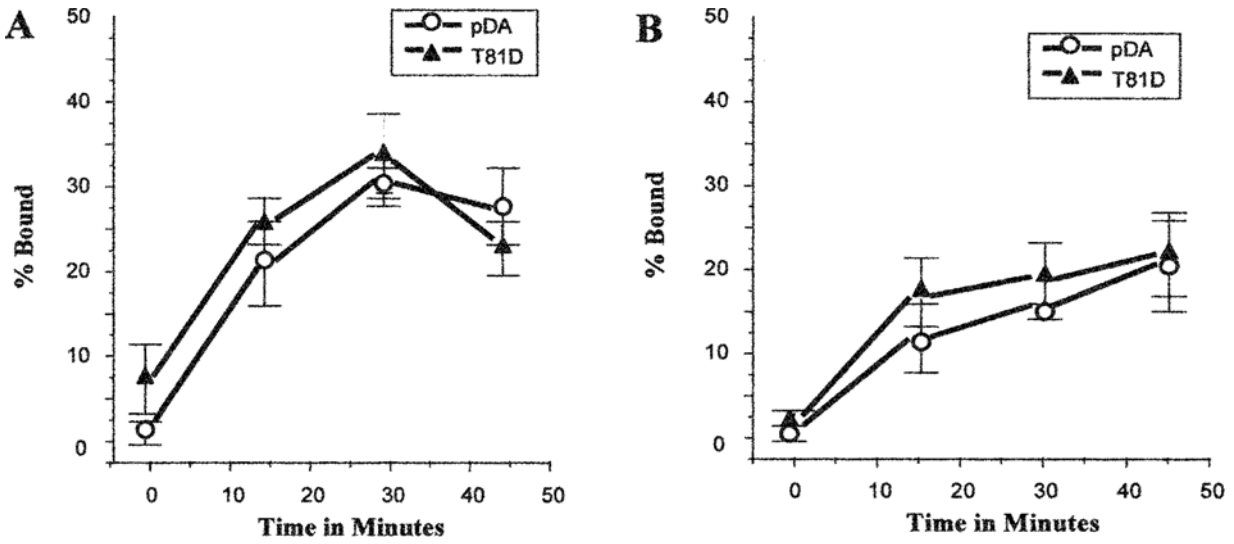

Figure 1 Binding of pDA (○) and T81D (▲) viruses to BHK-21 cells (**A**) and L929 cells (**B**). Radiolabeled virus was added to precooled cell monolayers at 4°C. At indicated time points, unbound virus was removed, the cell monolayers washed, cell-associated virus was counted in a liquid scintillation counter and the percent bound calculated. The data represent the mean ± standard error of two experiments of duplicates. We found no difference between the two viruses in binding to either BHK-21 (**A**) or L929 (**B**) cells.

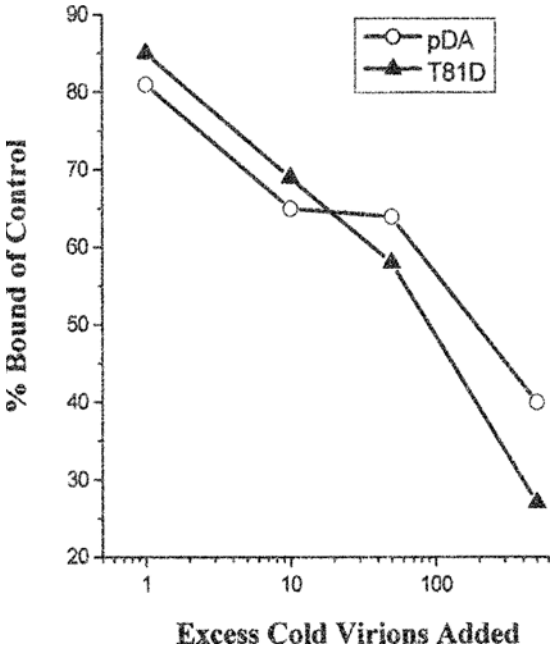

Figure 2 Competitive binding curves of pDA (○) and T81D (▲) viruses to BHK-21 cells. A constant amount of radiolabeled virus was incubated with increasing amounts of unlabeled pDA virus on BHK-21 cells for 30 min at 4°C. Unbound virus was removed, cell monolayers washed, and the cell-associated virus was counted in a liquid scintillation counter and the percent inhibition of control was calculated. Binding of both radiolabeled pDA and T81D viruses were equally inhibited with cold pDA virus.

virus by gradient sedimentation. The first observation was that wild-type pDA uncoated like other cardioviruses. We observed that the peak corresponding to whole virus particles (Figure 5, fractions 11–13) did not shift or produce a shoulder that would indicate a change in size from a 160S to a 135S subunit (Vrijsen *et al*, 1993).

To determine if there was a difference between the uncoating of pDA and T81D, we examined the rate of reduction of radioactivity in the 160S peak. Preliminary experiments with pDA virus indicated that the optimal incubation time for analysis of uncoating was 40 min at 37°C. After 40 min at 37°C, both virus infections showed reduced cell-bound radioactivity in the 160S peak (Figures 5 and 6). The data from two experiments indicate that both viruses showed an 80% decrease in the bound radioactivity. This reduction is most likely due to decapsidation of virions and elution of viruses from the cell surface. In picornavirus infection, a significant number of virions are known to elute from the cell surface after transfer from 4°C to 37°C (Baxt and Bachrach, 1980). We confirmed the significant elution of pDA and T81D virions from the cell surface to the supernatant after 40 min at 37°C (data not shown).

T81D mutant displays a delayed rate of RNA release in BHK-21 cells To measure RNA release, the step at which the viral RNA is released from the viral capsid, we monitored the loss of photosensitivity of virions grown in the presence of a chromogenic dye. For this assay, virus stocks were prepared in the presence of NR; the resulting NR virus was able to initiate an infection normally as long as all of the elements of infection were kept in the dark. The period of light sensitivity has been correlated with the time required for the virus to release its RNA into the cells. After RNA release, the NR molecules are no longer in close contact with the RNA with which they are encapsidated, and thus the RNA is no longer susceptible to irradiation (Kirkegaard, 1990).

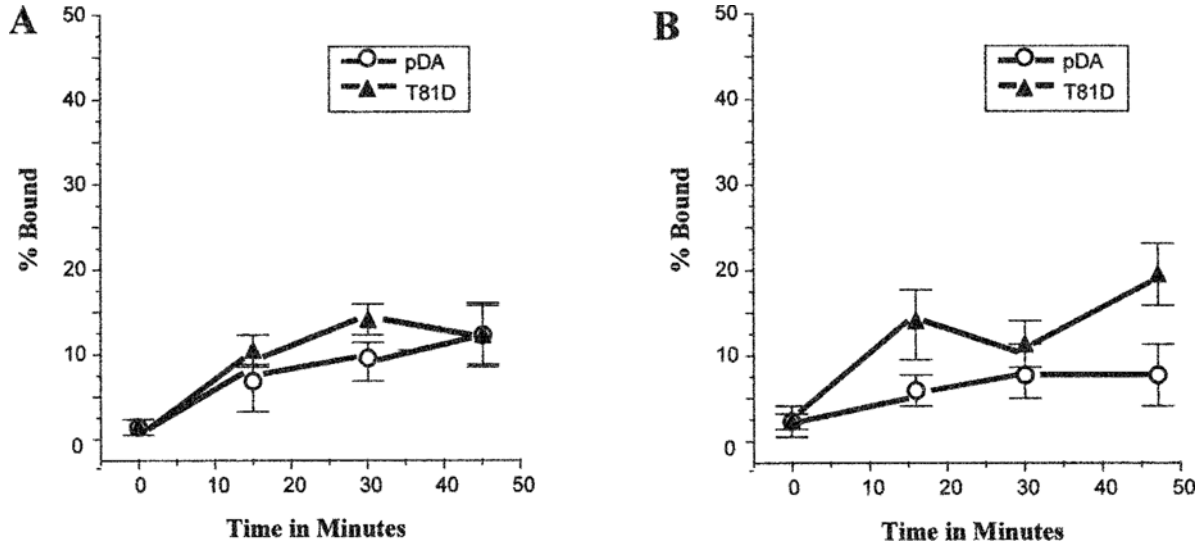

Figure 3 Binding of pDA (○) and T81D (▲) viruses to CNS derived cells. Radiolabeled virus was added to precooled cell monolayers of Neuro-2a cells (**A**) and astrocytes (**B**) at 4°C. At indicated time points, unbound virus was removed, the cell monolayers washed, cell-associated virus was counted in a liquid scintillation counter and the percent bound calculated. The data represent the mean ± standard error of two experiments of duplicates. Although low levels of viral binding was observed with either Neuro-2a cells (**A**) or astrocytes (**B**), no difference was seen in the binding between pDA and T81D viruses.

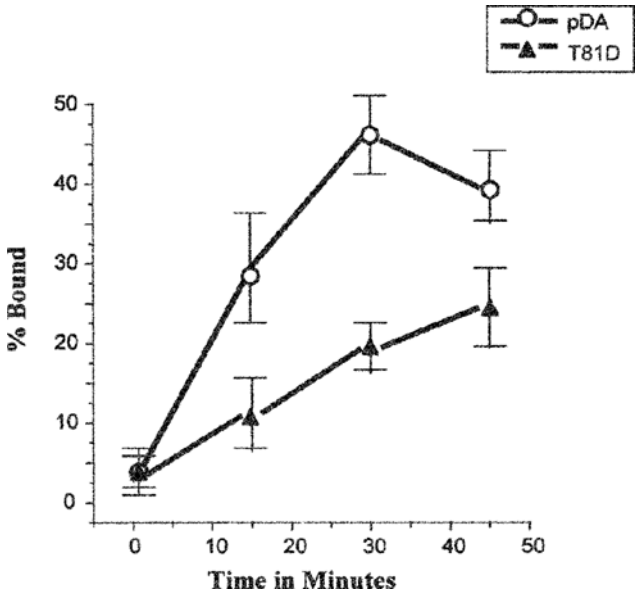

Figure 4 Binding of pDA (○) and T81D (▲) viruses to BSC-1 cells. Radiolabeled virus was added to precooled cell monolayers at 4°C. At indicated time points, unbound virus was removed, the cell monolayers washed, and the cell-associated virus was counted in a liquid scintillation counter and the percent bound calculated. The data represent the mean ± standard error of two experiments of duplicates. pDA virus bound more efficiently than T81D mutant virus.

We monitored the escape of the viral RNA from NR labeled virions (Figure 7). NR-labeled pDA and T81D viruses were prebound to monolayers of BHK-21 cells at 4°C; the cell monolayers were then washed twice and shifted to 37°C for various periods of time. All manipulations were performed under low levels of red light. With increasing periods of incubation at 37°C, increasing amount of RNA escaped photoinactivation; by 40 min 80% of the NR-pDA virus was immune to irradiation. However, the amount of NR-T81D virus immune to irradiation increased slowly; the difference was seen most clearly at the 40- and 60-minute time points. Thus, NR-T81D virus showed delayed release of its RNA compared to that of NR-pDA.

Neutralization of cell-free (STAN) and cell-associated (PAN) viruses by anti-TMEV antibody

Anti-virus antibody is known to neutralize not only virus in suspension (STAN) but also virus bound to the surface of host cells (PAN). We previously demonstrated that pDA virus can persistently infect mice despite high serum anti-TMEV antibody titers, whereas T81D virus-infected mice mount moderate anti-TMEV antibody responses and can eradicate virus thus preventing the virus from disseminating within the CNS (McCright *et al*, 1999). We compared the susceptibility of pDA and T81D viruses to STAN and PAN. First, we determined the

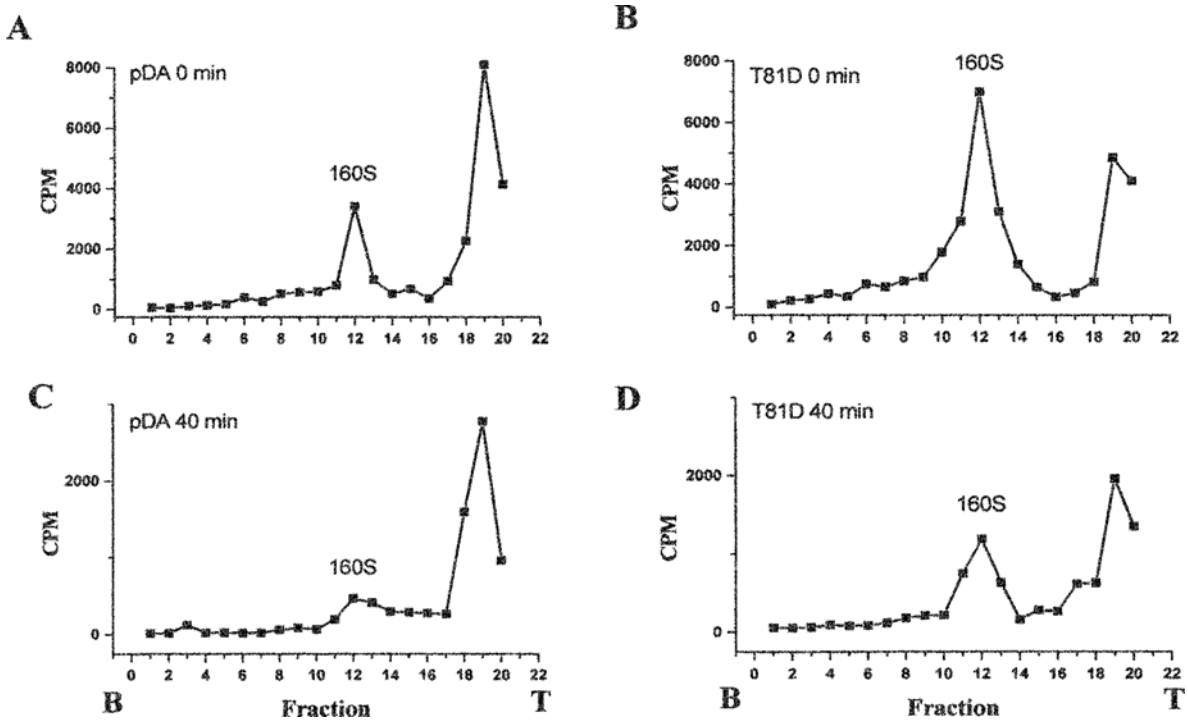

Figure 5 pDA and T81D viruses uncoat without the production of intermediates. pDA virus (**A**, **C**) or T81D virus (**B**, **D**) were added to precooled cell monolayers and allowed to bind. The cells with bound virus were lysed immediately (**A**, **B**) or after a 40-min (**C**, **D**) incubation time at 37°C to allow for uncoating. The lysate was separated in 15–30% sucrose gradients and fractions were counted for radioactivity. The top (T) and bottom (B) of the gradients are indicated. Intact virions sediment in fractions 11–13. A representative experiment is shown.

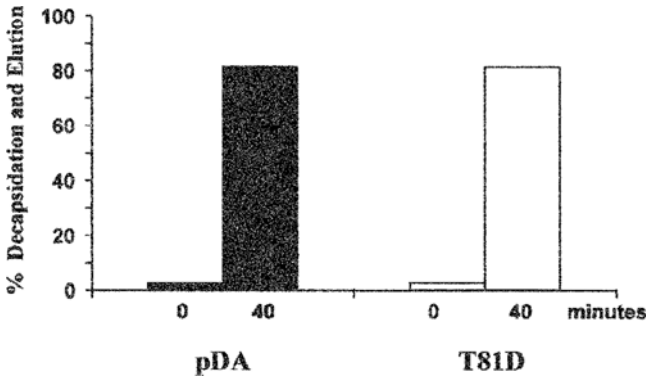

Figure 6 Comparable uncoating between pDA and T81D viruses. The decrease in radioactivity in the 160S peaks are similar between pDA and T81D viruses. Uncoating was set at 0% at 0 min postbinding. After 40 min at 37°C, both virus infections showed an 80% loss in cell-bound radioactivity in the 160S peak. The data represent an average of two experiments. There was <3% variation between pDA and T81D at the 40-minute time points.

ability of different antibodies to neutralize viruses in suspension (STAN) (Table 1). All three antibodies—H5 (control), H7, and H8—recognize VP1 on Western blots (Fujinami *et al*, 1988). The epitopes on VP1 of H5 and H8 are unknown, while the H7 antibody recognizes loop II of VP1 as its epitope. The H8 monoclonal antibody completely neutralized both viruses up to a dilution of 1:5000, whereas the H7 monoclonal antibody neutralized both viruses at 1:100 and 1:500 dilutions and exhibited different levels of neutralization at higher dilutions. The pDA virus was only 50% neutralized at a 1:1000

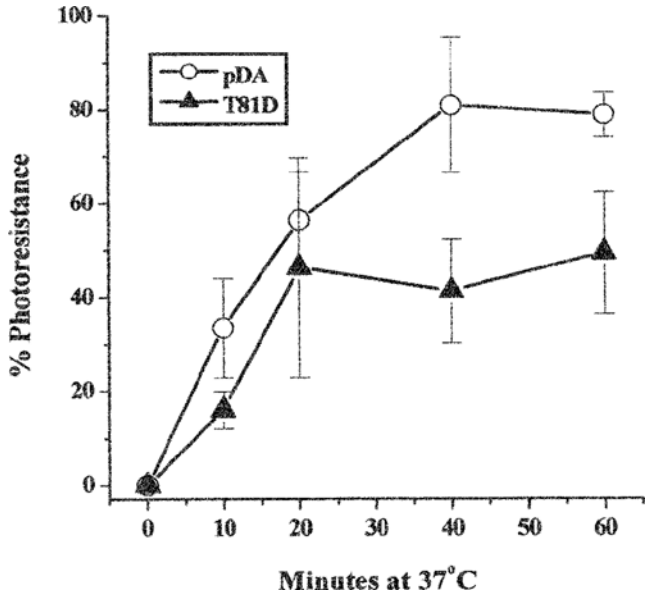

Figure 7 Time course of RNA release for wild-type and mutant viruses on BHK-21 cells. Increase in resistance to irradiation of pDA (○) and T81D (▲) viruses after the indicated time (in min) of incubation at 37°C. T81D virus had a reduced rate of RNA release, compared with that of pDA virus. The mean ± standard error of two experiments is shown.

Table 1 Percent neutralization by different monoclonal antibodies[a]

		Antibody dilution			
Virus	*Antibody*	*1:100*	*1:500*	*1:1000*	*1:5000*
pDA	H7	99	93	51	0
T81D	H7	96	97	93	67
pDA	H8	95	94	92	92
T81D	H8	98	95	96	94
pDA	H5	0	0	0	0
T81D	H5	0	0	0	0

[a]200 PFU of virus was incubated with various dilutions of antibodies, plaqued, and the percent neutralization calculated.

dilution and not at all at a 1:5000 dilution. In contrast, the T81D virus was completely neutralized with a 1:1000 dilution of H7 antibody and up to 67% of virus was neutralized at a 1:5000 dilution. The control monoclonal antibody H5 did not neutralize the viruses.

Next, we tested anti-TMEV monoclonal antibodies for its ability to effect PAN, that is, to reduce the number of plaques when added to cells that had already adsorbed, but not yet internalized the virus. To this end, monolayers of BHK-21 cells were infected with viruses at 4°C for 30 min, washed, and shifted to 37°C. We added monoclonal antibodies at the indicated times (Figure 8). After removing unbound antibody, the cells were treated as usual for a viral plaque assay. As seen in Figure 8, when H7 or H8 antibodies were added immediately after completion of virus attachment but before internalization (0 min), both pDA and T81D viruses showed similar resistance to PAN; 10% and 40% of inoculated viruses survived after treatment with H8 and H7 antibodies, respectively. In T81D virus infection, both antibodies showed efficient PAN even at 40 and 60 min at 37°C. In contrast, pDA virus developed resistance and 90% of viruses were resistant to PAN after 40 min at 37°C. Similar data using polyclonal anti-TMEV antibody were obtained (data not shown).

Discussion

We previously found that the T81D mutant virus exhibited a varied phenotype in both *in vitro* and *in vivo* studies (McCright *et al*, 1999). In this paper we have addressed the hypothesis that the change of amino acid 81 of loop I of VP1 from a threonine to an aspartate was responsible for altered virus-cell interactions and/or virus neutralization leading to an altered phenotype.

The data from the binding experiments suggested that this step of virus entry was not responsible for the phenotypic difference seen between pDA and T81D viruses. The T81D mutant bound BHK-21, L929, Neuro-2a, and astrocyte cell lines to a similar extent as wild-type pDA virus, although a difference

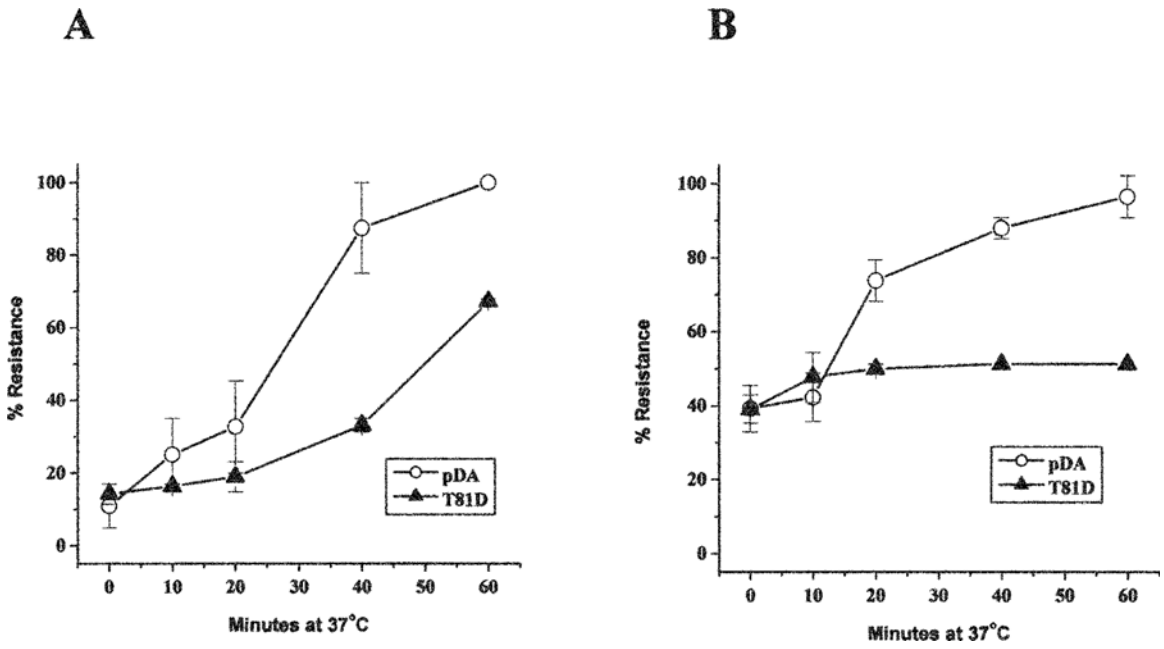

Figure 8 Postadsorption neutralization (PAN) of pDA (○) and T81D (▲) viruses by monoclonal antibody H8 (**A**) and H7 (**B**). Virus was allowed to adhere to precooled BHK-21 cell monolayers for 30 min at 4°C. Cell monolayers were washed and placed at 37°C. Antibodies were added at indicated time points. Percent resistance was calculated compared with the amount of virus without the addition of antibody. Both pDA and T81D viruses showed similar resistance to PAN at 0 min; 10% and 40% of inoculated viruses survived after treatment with H8 and H7 monoclonal antibodies, respectively. In T81D virus infection, both antibodies showed efficient PAN even at 40 and 60 min at 37°C. However, pDA virus developed resistance and 90% of viruses were resistant to PAN after 40 min at 37°C. The mean ± standard error of two experiments of duplicates is shown.

was seen with the semipermissive cell line BSC-l. Furthermore, both viruses similarly bound to receptors on BHK-21 cells as indicated by the competitive inhibition data. The change in the amino acid at the top of loop I of VP1 did not affect binding, at least in the permissive and neuronal cell lines. We cannot exclude the possibility that *in vivo* differences in binding to glial cells or neurons occurred that resulted in the altered disease phenotype.

Analyses of the uncoating experiments showed that TMEV uncoated like EMC virus, which belongs to the genus *Cardiovirus*. There were no stable intermediates detected upon uncoating. Virions disassociated into smaller fragments without evidence of stable A particles or empty capsids. When the rates of capsid dissociation/elution were examined for pDA and T81D viruses, no difference in the rates were found between the pDA and T81D viruses.

A difference was observed in the rate of RNA release into cells between the two viruses. The T81D mutant had a slower rate of RNA release. Therefore, the initial virus-cell interactions appear to be the same for these viruses, but the final step of uncoating differed. Studies with mengovirus mutants have found that viruses might have differences in binding, uncoating, or RNA release that are mutually exclusive (Mak *et al*, 1970). Even though a difference in uncoating was observed, no difference in RNA release was seen with some mengovirus mutants.

The neutralization data indicated that the H8 antibody neutralized both viruses in suspension up to a dilution of 1:5000, whereas the H7 antibody showed a different pattern of neutralization. H7 antibody could neutralize the T81D mutant to a greater extent than pDA virus. This antibody recognizes loop II of VP1 as part of its epitope. The change in loop I of the T81D mutant rendered it more susceptible to neutralization, perhaps by affecting the tertiary structure of the epitope recognized by the H7 antibody making it more accessible for the antibody.

We also demonstrated that anti-TMEV antibodies can neutralize viruses after completion of virus binding as has been reported for poliovirus infection (PAN) (Vrijsen *et al*, 1993). Although a precise mechanism of PAN is not clear, antibody-mediated stabilization of virion structure has been proposed leading to inhibition of neutralization in poliovirus infection (Vrijsen *et al*, 1993). Before the initiation of capsid dissociation and RNA release (or internalization) of viruses (0 min at 37°C), both pDA and T81D viruses showed similar susceptibility to PAN either by H7 or H8 antibody. During T81D virus infection, both antibodies showed efficient PAN even at 40 and 60 min at 37°C. In contrast, pDA virus developed resistance and 90% of virions were resistant to PAN after 40 min at 37°C. The different kinetics of

antibody resistance between pDA and T81D viruses are similar to the different kinetics of RNA release between NR-labeled pDA and T81D viruses. This supports the hypothesis that capsid dissociation with RNA release occurs on the plasma membrane without endocytosis in TMEV infection. Wilson and Cooper (1963) demonstrated that the rate of RNA release was the same as the rate of gain of antiserum resistance in NR-labeled poliovirus infection, where uncoating was proposed to occur on the plasma membrane. Therefore, the slow uncoating step in T81D virus would favor neutralization by anti-TMEV antibodies, while rapid uncoating by pDA favors resistance to PAN. During reovirus infection, protective antibodies have been demonstrated to inhibit postbinding events, including internalization and uncoating (Virgin *et al*, 1994).

The change in loop I of VP1 had little affect on binding or the manner of virus elution/decapsidation, but did have a major affect on RNA release and neutralization. How this can be explained is not yet clear. For polioviruses it has been stated that the receptor plays a dual function in binding and release of the viral genome (Zajac and Crowell, 1969). Therefore, the initial interaction is not affected, but a downstream event of the virus-receptor interaction could have been affected.

The finding that there are differences in RNA release into the cells and neutralization between wild-type and T81D viruses could explain the difference in persistence *in vivo*. Serum neutralizing antibody might interfere with virus-cell interactions, particularly during the chronic phase of TMEV infection when anti-viral antibody titers are high. Anti-viral antibodies have been shown to inhibit both growth and spread of virus within the CNS for several neurotropic viral infections (Tyler *et al*, 1989). In T81D virus infection, slow RNA release and high susceptibility to neutralization by antibodies would result in a failure to establish virus persistence *in vivo*. Conversely, rapid RNA release and resistance to neutralization might favor virus persistence in wild-type DA virus infection.

Materials and methods

Cells and viruses BHK (baby hamster kidney)-21 cells and primary murine SJL/J astrocytes were maintained in Dulbecco's modified Eagles medium (DMEM, Gibco, Gaithersburg, MD). L929 (mouse fibroblast), BSC-1 (African green monkey kidney cell line), and Neuro-2a (mouse neuroblastoma) cells were maintained in complete minimal essential medium (MEM, Gibco). pDA virus is the wild-type DA virus of TMEV and is derived from pDAFL3, a plasmid that contains the entire DA virus genome and was kindly provided by Raymond P. Roos (University of Chicago) (Roos *et al*, 1989). A mutant virus, T81D, with a threonine to aspartate substitution in loop I of VP1, was generated by *in vitro* site-directed mutagenesis of pDAFL3 as described previously (McCright *et al*, 1999).

Radiolabeling of virus and purification Radiolabeled pDA and T81D viruses were made as follows. Subconfluent BHK-21 monolayers on 150-mm plates were infected at a multiplicity of infection (MOI) of 0.5–1.0 with either pDA virus or T81D virus in 6 ml of complete DMEM supplemented with 2% fetal bovine serum (FBS) (Gibco). After an hour adsorption period, additional media was added and the infection was allowed to proceed for 2 h. The media were removed and the infected monolayers washed twice with DMEM deficient in methionine and cysteine (Gibco) and incubated in the same media for 1 h to deplete internal methionine and cysteine pools. Media were then removed and DMEM deficient in methionine and cysteine supplemented with 2% FBS and 22 μCi/ml Trans^{35}S-label (ICN Pharmaceuticals, Inc, Costa Mesa, CA) was added. The infection was allowed to proceed until extensive cytopathic effects were seen. Cells were scraped and the entire contents of the plates were transferred to tubes and frozen and thawed three times and centrifuged for 15 min at 1500 rpm at 15°C to remove debris. To the supernatant 1/100 volume 10% NP-40 (USB, Cleveland, OH), and 1/1000 volume β-mercaptoethanol (β-ME) (Sigma, St. Louis, MO) were added and virus was pelleted in a Beckman type-35 rotor at 30,000 rpm for 1 h at 15°C. Virus was resuspended in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (Sigma) and 0.1% β-ME, and to this 10% Na-Sarkosyl (Sigma) and 20 mM EDTA (J. T. Baker, Phillipsburg, NJ) were added to a final concentration of 0.05%. The resultant material was put onto 20–50% CsCl (Gibco) gradients in PBS. After centrifugation at 37,000 rpm in a Beckman SW4l rotor at 4°C for 4 h, the virus bands were harvested. Virus was diluted in 10 ml PBS and pelleted at 27,000 rpm in a SW41 rotor for 1 h at 4°C. The virus pellets were resuspended in PBS containing 10% FBS and stored at −70°C. Radiolabeled viruses were titered by plaque assay (McCright *et al*, 1999).

Binding assays Similar numbers of BHK-21, L929, astrocytes, Neuro-2a, and BSC-l cells were chilled in six-well plates (Corning Costar, Cambridge, MA) for 30 min at 4°C. In duplicate wells, radiolabeled pDA and T81D viruses (in 0.5 ml media supplemented with 2% FBS) were added at an MOI of 20 and incubated at indicated time points. At the time points unadsorped virus was removed, the monolayers were washed with media supplemented with 2% FBS, and lysed with 0.5 ml 0.1 N NaOH (Fisher Scientific, Denver, CO). The cell lysate was transferred to scintillation vials; 3 ml EcoLume (ICN) was added and the samples were counted in a liquid scintillation counter. The experiments were performed twice and the percent bound was calculated as follows: (cpm of sample/input cpm) × 100. The means with the standard errors were graphed. For

competitive inhibition studies, precooled BHK-21 cells were incubated with radiolabeled virus in the presence of an equal amount, or 10-, 50-, or 500 times excess cold pDA virus for 30 min or without cold virus (control). Samples were then processed as before. Percent control was calculated as follows: (cpm sample/cpm control) × 100, where the control represents virus bound to BHK-21 cells for 30 min in the absence of cold virus.

Uncoating assays An adaptation of the procedures used by Calvez *et al* (Calvez *et al*, 1995) was followed. Briefly, subconfluent BHK-21 cell monolayers in 60-mm plates were precooled at 4°C for 30 min. Radiolabeled virus was added at an MOI of 250 in 0.75 ml DMEM (2% FBS) and allowed to adsorb for 30 min at 4°C. Cell monolayers were washed twice with cold DMEM (2% FBS) to remove unbound particles. DMEM (2% FBS) was added and the cells allowed to incubate for 0 or 40 min at 37°C. Cell monolayers were washed with DMEM (2% FBS) to remove eluted virus and virus fragments. Cells were harvested by scraping, resuspended in buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0), and incubated at 4°C for 10 min. Cells were lysed by the addition of 1/10 volume of lysis buffer (1% sodium dodecyl sulfate, 10% NP-40 in same buffer as before). After incubation on ice for 15 min, the cell lysates were centrifuged in a microfuge at 12,000 × g at 4°C. The clarified supernatants were layered onto 15–30% (w/v) sucrose (Sigma) gradients in PBS. Gradients were centrifuged in a SW41 rotor for 75 min at 40,000 rpm at 4°C. Twenty-two fractions (0.5 ml) were collected from the bottom of each gradient and 0.4 ml of the fractions was added to 3 ml EcoLume and samples were counted in a liquid scintillation counter. The percent decapsidation/elution was calculated as follows: (the total cpm of the peak fractions that contained whole virus after 40 minutes/total cpm of the peak fractions that contained whole virus at 0 min) × 100.

RNA release assay NR-labeled virus was prepared, assayed, and irradiated as described by Kato and Eggers (1969) and Kirkegaard (1990). Monolayer cultures of BHK-21 cells were grown in the usual growth medium that was then replaced with media containing NR (10 μg/ml). After 24 h, the cultures were infected either with pDA or T81D virus. The media containing NR was added and incubated overnight. The cells and medium were collected, frozen and thawed three times. Viruses were passaged in the presence of NR three times. All manipulations involving NR-labeled virus were conducted under a red light that had been shown to be noninactivating. Then, 200 plaque forming units (PFUs) of each NR-labeled virus was added to BHK-21 monolayers for 30 min at 4°C. The cell monolayers were washed twice and were irradiated by a 75-W white lamp at a distance of 10 cm for 10 min after various times of incubation at 37°C. A large plastic dish filled with water was placed between the lamp and the Petri dish to absorb radiant heat. Control plaque assays of the NR viruses, which received no irradiation, were conducted in parallel. The data were quantified by counting the numbers of plaques on the irradiated plates. The percentage of photoresistant virus was calculated using the following equation:

% resistance
= (the number of plaques on the irradiated plate after the indicated time − the number of plaques on the plate irradiated at 0 min)/ (the number of plaques on the nonirradiated control plate at 0 min) × 100.

Neutralization assay H5, H7, and H8 are monoclonal antibodies that recognize VP1 by Western blot (Fujinami *et al*, 1988). H7 and H8 are neutralizing antibodies and H5 is a nonneutralizing antibody. The H7 antibody recognizes loop II of VP1. To determine whether H8 and H7 monoclonal antibodies could effectively neutralize T81D mutant, a neutralization assay was performed. Viruses were diluted to 200 PFU in 0.1 ml DMEM (2% FBS) and were incubated with a 0.1 ml of 1:100, 1:500, 1:1000, or 1:5,000 dilution of monoclonal antibodies H7 or H8 in duplicate. The antibody-virus mixture was incubated on ice for 1 h. The mixture was then added to subconfluent BHK-21 cells in six-well cluster plates and plaqued as described previously (McCright *et al*, 1999). For controls, virus and cells incubated without added antibody or H5 monoclonal antibody (nonneutralizing) was used. Percent neutralization was calculated as follows:

100 − [(the number of plaques of sample/the number of plaques without antibody) × 100].

PAN PAN was measured using adaptations of the protocol used by Mak *et al* (1970) and Vrijsen *et al* (1993). Subconfluent BHK-21 cells in six-well plates were precooled at 4°C for 15 min. Duplicate sets of plates were infected with 200 PFU pDA and T81D viruses in 0.5 ml DMEM (2% FBS). After incubation for 30 min to allow for virus binding, unattached virus was removed by two washes with ice-cold DMEM (2% FBS). Prewarmed media was added and cells were put at 37°C. At 0, 10, 20, 40, and 60 min thereafter, H8, H7, or hyperimmune polyclonal rabbit anti-DA virus antibodies were added. The final dilution of each monoclonal antibody was: H7, 1:500; and H8, 1:333. After a 60-minute incubation at 37°C, the plates were washed once with DMEM (2% FBS) and overlaid with nutrient agar (plaque assay). The percentage of resistant viruses was calculated as follows:

% = (PFU at indicated time)/(PFU without antibody) × 100.

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