An Avirulent Mutant of Rabies Virus Is Unable To Infect Motoneurons In Vivo and In Vitro

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An antigenic double mutant of rabies virus (challenge virus standard [CVS] strain) was selected by successive use of two neutralizing antiglycoprotein monoclonal antibodies, both specific for antigenic site III. This mutant differed from the original virus strain by two amino acid substitutions in the ectodomain of the glycoprotein. The lysine in position 330 and the arginine in position 333 were replaced by asparagine and methionine, respectively. This double mutant was not pathogenic for adult mice. When injected intramuscularly into the forelimbs of adult mice, this virus could not penetrate the nervous system, either by the motor or by the sensory route, while respective single mutants infected motoneurons in the spinal cord and sensory neurons in the dorsal root ganglia. In vitro experiments showed that the double mutant was able to infect BHK cells, neuroblastoma cells, and freshly prepared embryonic motoneurons, albeit with a lower efficiency than the CVS strain. Upon further incubation at 37°C, the motoneurons became resistant to infection by the mutant while remaining permissive to CVS infection. These results suggest that rabies virus uses different types of receptors: a molecule which is ubiquitously expressed at the surface of continuous cell lines and which is recognized by both CVS and the double mutant and a neuron-specific molecule which is not recognized by the double mutant.

Tissue tropism of a virus is first determined by the interaction of viral surface protein(s) with molecules expressed at the surface of target cells. Expression of such molecules in a limited group of differentiated tissues restricts the tropism of a virus. Rabies virus is a clear example of such a situation. This enveloped virus, whose genome is a nonsegmented negative-strand RNA, belongs to the rhabdovirus family. The genome codes for five proteins, one of which, glycoprotein (G), is exposed at the surface of the virion (29). In vitro, the virus is able to infect various types of cells (19). In vivo its tropism is mostly restricted to neurons. However, after intramuscular inoculation, rabies virus can simultaneously infect neurons and muscle cells (15). Replication in muscle cells, which is observed particularly with virus can simultaneously infect neurons and muscle cells (15). Replication in muscle cells, which is observed particularly with

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and its inability to invade the mouse NS after intramuscular inoculation.

MATERIALS AND METHODS

Cells and virus. BSR cells, derived from baby hamster kidney (BHK) cells, were grown in Glasgow’s modified minimal essential medium supplemented with 10% calf serum at 37°C in a 5% CO2 incubator. NG108-15 (mouse neuroblastoma N18 × rat glioma C6) cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL) plus 10% fetal bovine serum (17).

Primary cultures of embryonic rat motoneurons. Spinal cord motoneurons were prepared according to the technique described by Ternaux and Portalier (Diagnostic Pasteur, Paris, France). For the pathogenicity tests, five mice were intracerebrally injected with 30-μl aliquots containing 10^7 PFU of each virus. Animals were kept under observation for 21 days. The surviving animals were challenged at day 28 by the intramuscular route with 50 μl of CVS containing 10^5 50% lethal doses (LD50).

The penetration experiments were conducted according to procedures already described (6). Briefly, 10 μl (10^5 PFU) of virus was injected into the forelimb. Animals were sacrificed 30 h later. The thoracic portion of the spinal cord and the corresponding dorsal root ganglia ipsilateral to the inoculated member were dissected as one piece and directly frozen in dry ice. Serial sections of tissues were made with a cryostat microtome (Instrument Company Ltd., Huntington, England) and recovered on gelatin-treated slides. They were fixed with acetone and treated with fluorescein isothiocyanate-conjugated antinucleocapsid antibodies (Diagnostic Pasteur, Paris, France).

For the antigenic mutants, two viruses were fully pathogenic for adult mice. They proceeded for 72 h at 37°C in minimal essential medium supplemented with 2% calf serum. Supernatants were collected, centrifuged at a low speed to discard cell debris, and frozen in aliquots at −70°C. The titers were determined as already described (18). Viruses were concentrated according to a previously described procedure (21).

Antiglycoprotein MAbs 50AC1 and 50AD1 were selected for their resistance to neutralization by MAbs according to a previously described procedure (21). They were isolated from revertant clones of the Av010 mutant or from RK4 (21, 28).

RESULTS

Selection of an antigenic avirulent double mutant. All avirulent mutants isolated from different rabies virus strains carry a single substitution in the glycoprotein, where arginine 333 is replaced by cysteine, glutamine, glycine, leucine, methionine, or serine in CVS (21, 28), by isoleucine in Evelyn-Rokitnicki-Abelson (ERA) (8), and by glutamate or serine in street Alabama Duferin (SAD) (11). An antigenic mutant carrying a lysine at position 333 was selected from the SAD_hern strain. This mutant was avirulent in mice after intramuscular inoculation but retained residual pathogenicity by the intracerebral route (11), like a mutant isolated from the CVS strain (28).

These results suggest that a positive charge at position 333 might be important in the pathogenic process. A further basic amino acid, the lysine at position 330, is present in antigenic site III (Fig. 1). Two MAR mutants with a substitution at that position were selected with appropriate MAbs: F69, which has a threonine (28), and RK4, which has an asparagine (Table 1). These two mutants were fully pathogenic for adult mice. They were partially neutralized by MAbs 28AD2 and 50AD1, whereas

TABLE 1. Characteristics of CVS and mutants having one or two substitutions in antigenic site III

<table>
<thead>
<tr>
<th>Virus</th>
<th>Selecting MAb</th>
<th>Amino acid at position</th>
<th>LD50 i.c. (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVS</td>
<td>Lysine</td>
<td>Arginine</td>
<td>10</td>
</tr>
<tr>
<td>RK4</td>
<td>50AC1</td>
<td>Asparagine</td>
<td>10</td>
</tr>
<tr>
<td>RL1</td>
<td>248-8</td>
<td>Lysine</td>
<td>&gt;10^4</td>
</tr>
<tr>
<td>K4-5</td>
<td>50AD1</td>
<td>Asparagine</td>
<td>&gt;10^4</td>
</tr>
</tbody>
</table>

LD50 i.c., 50% lethal dose administered intracerebrally.
MAR mutants with a substitution at position 333 could not be neutralized by these antibodies.

We selected an additional mutation at position 333 from mutant RK4 by using MAb 50AD1. The resulting mutant, K4-5, carried two amino acid substitutions: lysine to asparagine and arginine to methionine at positions 330 and 333, respectively (Table 1). Intracerebral inoculation of K4-5 indicated that this mutant was nonpathogenic for adult mice. Control experiments with mutants having one or the other substitution, RK4 and RL1, showed that RK4 (K330N) was pathogenic and that RL1 was avirulent (R333M) (28) (Table 1).

Infection of the mouse NS after peripheral inoculation. In order to test if the presence of these two mutations in the glycoprotein gene had an effect on the early events of the infection in animals, the double and respective single mutants were intramuscularly injected into the forelimbs of adult mice. By this route of inoculation, it has been shown that AvO1 (the prototype of avirulent mutants) was able to infect motoneurons in the spinal cord and sensory neurons in the dorsal root ganglia as efficiently as CVS (6). Both viruses directly penetrated nerve endings without prior multiplication in the muscle at the site of inoculation. At 30 h after inoculation of a high virus dose (Table 2), the thoracic portion of the spinal cord and the corresponding dorsal root ganglia were dissected from infected animals. At this time, only primary infected neurons can be detected (6). Serial sections of both tissues were processed for detection of rabies virus antigens. Like the pathogenic viruses CVS and RK4, the avirulent mutant RL1 infected both motoneurons and sensory neurons during the first cycle of infection in the mouse NS (Table 2). All infected neurons were located ipsilateral to the inoculation site (data not shown). The double mutant, on the other hand, did not infect any neurons in four injected mice, either in the spinal cord or in the ganglia. A fifth mouse exhibited infection of only three neurons in one dorsal root ganglion (Table 2). It should be noted that these mice received a 10-fold-higher virus inoculum than did those injected with the CVS strain.

Infection of neuroblastoma cells by avirulent mutants. To test the capacity of the double mutant to multiply in continuous cell lines, BSR and NG108-15 cells were infected with CVS or antigenic mutant at an MOI of 5 or 6 PFU/cell. Virus supernatants were titrated on BSR cells (Fig. 2). Rabies virus-infected cells were detected by immunofluorescence 18 h after adsorption. At this MOI, 100% of BSR cells were infected with both viruses. K4-5 infected only 50% of NG108-15 cells, while CVS infected 100% of these cells, suggesting that the penetration of the mutant was slightly affected. In all cases, the intensities of the fluorescence 18 h after infection were comparable, an indication that protein synthesis proceeded at the same rate once penetration had occurred. With both viruses, production of virus was lower in neuroblastoma cells than in BSR cells. Production of mutant virus was also lower than that of CVS on both types of cells. For instance, at 48 h after infection, production of virus with K4-5 and CVS was equal, respectively, to 10 and 65 PFU/cell on NG108-15 cells and to 120 and 900 PFU/cell on BSR cells.

Progressive resistance of spinal motoneurons to infection by the double mutant. We decided to monitor the infectious process in primary cultures of spinal motoneurons. These motoneurons were isolated from the spinal cord of 15- to 17-day-old rat fetuses and purified by differential centrifugation as described in Materials and Methods. Under our conditions, where defined medium was changed daily, motoneurons could be maintained in cultures for up to 2 weeks (Fig. 3a). Immunocytochemistry analysis performed on these cells with an antiacetylcholine antibody indicated that this cell population synthesized acetylcholine (Fig. 3b). Coverslips were seeded with 3 × 10^4 to 3 × 10^5 motoneurons on a poly-L-lysine- and laminin-treated surface. These motoneurons were infected with CVS or the double mutant at 2, 6, and 10 days after plating. At that stage, about 10% of the motoneurons were still alive. The inoculum size was standardized to 4 × 10^6 PFU/coverslip, but the real MOI could not be calculated because the virus also attached to the laminin substrate. Infection was stopped 24 h later, and infected neurons were counted by double-labeling immunofluorescence. These cells were identified as neurons by immunocytochemistry (Fig. 4A and C). At any stage of infection, between 68 and 77% of cells were infected with CVS (Fig. 4B and 5). On the contrary, the double mutant could infect only 30% of motoneurons after 2 days in cultures (Fig. 5). Then, the resistance to infection increased with time, and after 10 days in cultures, less than 3% of motoneurons were infected with the double mutant (Fig. 4D and 5). A similar experiment was performed on mouse spinal motoneurons purified from 13-day-old embryos. Following 6 days in cultures, the mouse motoneurons were infected for 24 h with CVS or the double mutant as described for rat motoneuron infection. After immunocytochemical processing, the CVS-infected culture, 444 of 502 (88.4%) MAP2^+ motoneurons contained rabies virus antigens, while in the double mutant-infected culture, only 7 of 505 (1.4%) MAP2^+ motoneurons were positive for rabies virus antigens.

TABLE 2. Infection of mouse dorsal root ganglia (DRG) and spinal cord (SC) after peripheral inoculation of CVS and antigenic mutants^a

<table>
<thead>
<tr>
<th>Mouse</th>
<th>No. of infected neurons 30 h after inoculation of (PFU):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CVS (4 × 10^4)</td>
</tr>
<tr>
<td>DRG SC</td>
<td>DRG SC</td>
</tr>
<tr>
<td>1</td>
<td>48 80</td>
</tr>
<tr>
<td>2</td>
<td>41 87</td>
</tr>
<tr>
<td>3</td>
<td>18 9</td>
</tr>
<tr>
<td>4</td>
<td>15 13</td>
</tr>
<tr>
<td>5</td>
<td>25 53</td>
</tr>
</tbody>
</table>

^a Ten microliters of concentrated virus was inoculated into the right forelimbs of adult mice. Each group of two values was obtained from the same animal.

FIG. 2. One-step growth curves for CVS (●) and K4-5 (○) on BSR (—) and NG108-15 (– – –) cells. The MOIs were 5 (CVS) and 6 (K4-5). After 1 h of adsorption at room temperature, the inoculum was removed and cell layers were washed before the addition of the medium. Supernatants were changed after 4 h in order to remove desorbed virus. Incubation was done at 37°C, p.l., postinfection.
DISCUSSION

It has been well documented that rabies virus infects most continuous cell lines in vitro, while its tropism is restricted mostly to neurons in animals. In addition, pathogenic strains infect most neuronal populations in the CNS. Previous studies suggested the existence of two kinds of receptors used by rabies virus to attach to a target cell: ubiquitously distributed cell surface molecules and specific molecules present on a few cell lines and on some differentiated tissues (31). Ubiquitous receptors could be lipids and gangliosides (22, 23, 31), and a specific receptor could be the nicotinic acetylcholine receptor (13). However, since this complex is not present on many categories of neurons permissible for rabies virus, it cannot be the only receptor which mediates viral entry into neurons.

Previous work on the fixed strain CVS showed that point mutations in the glycoprotein gene leading to the replacement of arginine at position 333 generated avirulent viruses with a restricted capacity to propagate in the CNS (10, 12). These viruses were able to penetrate motoneurons and sensory neurons as efficiently as the original CVS strain after intramuscular injection, yet transneuronal transfer of the mutant viruses did not occur (6). On the contrary, the double mutant K4-5 (K330N-R333M) described here infected neither sensory neurons nor motoneurons by this route of inoculation. Since a mutation in the viral glycoprotein likely influences virus entry

FIG. 3. Purified rat embryonic spinal motoneurons in cultures. (a) Cultures of dissociated motoneurons were grown on glass coverslips coated with poly-L-lysine (0.005%) and laminin (0.001%) in the presence of DMEM-F12 medium with additives. Living cells were observed by phase-contrast microscopy 6 days after plating. (b) Immunoperoxidase detection of acetylcholine in the same culture of motoneurons after fixation with paraformaldehyde. Bar, 15 μm.

FIG. 4. Infection of spinal motoneurons from rat embryos after 10 days in cultures with CVS or antigenic double mutant K4-5. Cells were plated on glass coverslips treated with poly-L-lysine and laminin, kept for 10 days, and then infected for 24 h with CVS (A and B) or K4-5 (C and D). Cultures were fixed and permeabilized before simultaneous detection of neurons (A and C) and viral antigens (B and D) by double-labeling immunofluorescence. Bar, 30 μm.
infection with mutant K4-5 in vitro. Motoneurons were seeded on poly- L-lysine- and laminin-treated glass coverslips at a density of 3 × 10⁴ to 3 × 10⁵ cells per coverslip, depending on the culture time. They were infected at day 2, 6, or 10 with CVS or K4-5 for 24 h at 37°C. After fixation and permeabilization, neurons were identified with an anti-MAP2 mouse MAb, and infected cells were detected with a rabbit anti-rabies virus nucleocapsid antibody. The percentage of infected motoneurons was estimated as the ratio of the number of doubly-labeled cells to the number of MAP2-labeled cells (value estimated from 10⁵ MAP2⁺ cells counted). The experiment was performed in quadruplicate.

FIG. 5. Rat embryonic spinal motoneurons acquire progressive resistance to infection with mutant K4-5 in vitro. Motoneurons were seeded on poly-L-lysine- and laminin-treated glass coverslips at a density of 3 × 10⁴ to 3 × 10⁵ cells per coverslip, depending on the culture time. They were infected at day 2, 6, or 10 with CVS or K4-5 for 24 h at 37°C. After fixation and permeabilization, neurons were identified with an anti-MAP2 mouse MAb, and infected cells were detected with a rabbit anti-rabies virus nucleocapsid antibody. The percentage of infected motoneurons was estimated as the ratio of the number of doubly-labeled cells to the number of MAP2-labeled cells (value estimated from 10⁵ MAP2⁺ cells counted). The experiment was performed in quadruplicate.

or egress but not viral replication, these data suggest that the block occurs at an early step of the viral life cycle, such as binding, endocytosis, or fusion, reflecting a lack of interaction between the mutated glycoprotein and a neuronal component.

Using primary cultures of motoneurons, we were able to extend data obtained in the animal experiments. These neurons were isolated from rat embryonic spinal cord and were considered to be motoneurons on the basis of several criteria (see Results). In these cultures, we found that motoneuron susceptibility to K4-5 decreased to less than 3% after 10 days. Thus, the more the motoneurons survive in cultures, the more they are resistant to K4-5. This situation is reminiscent of what was observed for mice with mutants having a substitution at position 333 of the glycoprotein. Such mutants are still pathogenic for baby mice and multiply in their brains like the wild type (28). They lose the capability to invade the NS within the first 3 weeks after birth (unpublished data).

Experiments with established cell lines, BSR or neuroblastoma, did not reveal dramatic differences between CVS and K4-5, although the mutant was slightly affected in its capability to penetrate NG108-15 cells (at an MOI of 6, only 50% of the cells were infected). Although the fluorescence of infected cells was of a similar intensity, viral production was lower with K4-5 than with CVS, probably because maturation and/or virus stability was affected by the mutations. With both viruses, viral production was lower in neuroblastoma cells. However, the multiplication of K4-5 and CVS in in vitro cell cultures was not dramatically different. Once again, this observation illustrates the difficulty in the use of any kind of established cell line to identify specific neuronal rabies virus receptors, because such cells express ubiquitous receptors. Recently, this problem was partly circumvented by the use of a binding assay with Sf21 insect cells, expressing the rabies virus glycoprotein at their surface, and several established cell lines. We demonstrated that only neuronal cell lines (including the one used in these studies) expressed specific binding sites for the rabies virus glycoprotein and that the two mutations present in K4-5 abolished this binding (28a). These binding sites might be “high-affinity” neuron-specific receptors for rabies to which the mutated glycoprotein could not bind (or to which it bound with a lower affinity insufficient to mediate the attachment of lepidoptera cells to neuroblastoma cells).

K4-5 differs from CVS by two amino acid substitutions in the ectodomain of the glycoprotein. These changes are located at positions 330 and 333, previously identified as belonging to antigenic site III (21). A partial amino acid sequence comparison of several laboratory and wild-type strains showed an almost complete conservation of antigenic site III (Fig. 1). Antigenic site III has been defined as a continuous and conformational site (4). This domain could be delineated by (i) a potential glycosylation site at position 319 (Fig. 1), which is present in all strains (1–3, 5, 27, 32), and which has been demonstrated to be used in CVS (30), and (ii) a proline at position 340 (this amino acid is known to cause bending in the secondary structure of proteins). Another argument in favor of the structural importance of this proline is the presence of the minor site a. Antigenic mutations at this site have been located at positions 342 and 343, and no change in the sensitivity of MAR mutants from minor site a and MAbs from site III, and vice versa, has been observed (4). Moreover, the structural organization of site III is also conserved in the glycoproteins of Mokola virus (26). This finding may be an indication of the importance of this region for the viability of the lyssaviruses.

The region from positions 320 to 340 contains two positively charged amino acids, with the exception of lysine at position 320, which is located in the Asn-X-Ser/Thr motif and which is probably masked by the sugar chain (Fig. 1). Substitution of these two residues with uncharged ones renders the virus unable to penetrate sensory neurons and motoneurons. Replacement of only one positively charged amino acid (in RK4 and AvO1 mutants) does not reduce the capacity of these mutants to infect these types of neurons, but in AvO1 it blocks transmission to interneurons (6).

The selection of antigenic mutants with a restricted capacity to infect neurons by use of neutralizing MAbs allowed the identification of two key amino acids for the penetration of rabies virus in neurons. It is likely that further key amino acids will also be implicated in the interaction of the viral glycoprotein with the cell receptor. The development of reverse genetics for the negative-strand RNA viruses and especially for rabies virus (20) has provided new tools for the study of amino acids implicated in interactions with the neuronal receptor(s).

On the other hand, K4-5, by its inability to infect motoneurons and sensory neurons, will be useful for the identification of neuronal receptors for rabies virus.

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