SHORT COMMUNICATION

Neutralizing Epitope on Penetration Protein of Vaccinia Virus

YASUO ICHIHASHI and MASAYASU OIE

Department of Virology, Faculty of Medicine, Niigata University, Asahimachi, Niigata 951, Japan

Received January 17, 1996; accepted April 4, 1996

The monoclonal antibody 2D5 neutralized vaccinia virus by preventing penetration of the virus and reacting with VP23-29K. The conformation of the VP23-29K was maintained by a disulfide bond(s), and the 2D5mAb reacted stronger with the nonreduced 23-kDa form than with the reduced 29-kDa form. We selected several escape mutants. Sequences of the A17L genes, which were thought to encode the VP23-29K, did not show cognate mutation. Genomic DNA of a 2D5mAb-resistant mutant (M4) was cleaved with HindIII, and all the fragments were introduced into parental IHD-J strain vaccinia virus by transfection. Only the L fragment produced a 2D5mAb-resistant virus. Dissection of the L fragment and subsequent transfection revealed that the L1R gene induced the 2D5mAb-resistant virus. The 2D5mAb-resistant mutants showed a consensus G to A conversion at nucleotide 101 of their LIRs which would replace asparatic acid 35 with asparagine. Ishibashi-111 strain mousepox virus spontaneously resistant to 2D5mAb also had the same sequence at this region. Moreover, the VP23-29K was myristoylated as predicted by the L1R gene. The coding gene of the VP23-29K was LIR.

Vaccinia virus infects cells by means of membrane fusion and induces syncyta at the late stage of infection. The inhibition of the membrane fusion phenomena by a monoclonal antibody (2D5mAb) suggests that its target antigen has an important role in the processes. The 2D5mAb reacts with nonreduced 23-kDa protein and with reduced 29-kDa protein of the virus, and we termed this protein VP23-29K (1, 2). N-terminal amino acid sequence analysis indicates its coding gene to be A17L. However, unlike the nonreduced 23-kDa form, the reduced 29-kDa protein reacted weakly with the 2D5mAb, and direct N-terminal amino acid sequencing of the 29-kDa protein did not produce signals. Recently the 2D5mAb was reported to react with L1R encoded protein that behaved like VP23-29K (3).

To further characterize the VP23-29K, we selected mutants that were resistant to neutralization by the 2D5mAb. Vero cells infected with the IHD-J strain of vaccinia virus were incubated in the presence of a mutagen (MNNG) to induce point mutations into the progeny virus (4). Mutant viruses were treated with trypsin to remove extracellular enveloped virus (EEV), because the EEV was resistant to neutralization (5). The activated viruses were selected by the 2D5mAb. After three repeated selective passages, the titer of the progeny virus was no longer reduced by exposure to the 2D5mAb. Three clones (M1, M2, and M4) were isolated and plaque purified. Cells infected with the M1 and M2 mutants as well as the parental IHD-J strain virus did not cause cell fusion, while the M4 mutant induced cell fusion at the late stage of infection.

The 2D5mAb-resistant mutants were tested using three anti-VP23-29K mAbs (Table 1). The M1, M2, and M4 mutant viruses were resistant to the 2D5mAb and G7H5mAb, whereas the 8C2mAb neutralized them with the same efficiency as the parental IHD-J strain virus. All three mAbs neutralized the CPR-C1 strain of cowpox and Copenhagen strain of monkeypox viruses, but the Ishibashi-111 strain of mousepox virus was resistant to the 2D5/G7H5mAb and sensitive to the 8C2mAb. The VP23-29K had two distinct neutralizing epitopes. One was reactive to 2D5mAb/G7H5mAb, and the other was defined by 8C2mAb. The 8C2-reactive epitope was conserved among a wide range of orthopoxviruses, whereas the 2D5/G7H5mAb-reactive epitope varied between the M1/M2/M4/mousepox and vaccinia/cowpox/monkeypox virus groups.

Structural proteins of the three 2D5mAb-resistant mutants were separated by SDS–PAGE and then immunoblotted against 2D5mAb and 8C2mAb (Fig. 1). Two vaccinia viruses (IHD-J and WR strains) and vaccinia mutants (D1 and vRB10), the CPR-C1 strain of cowpox, the Ishibashi-111 strain of mousepox, and the Copenhagen strain of monkeypox virus were examined in parallel. All the virus samples were adjusted to 20 μg/lane. Profiles of the viral proteins were revealed by silver staining the gel (Figs. 1A-1 and 1B-1) and by immunoblotting with polyclonal mouse anti-IHD-J strain vaccinia virus (Figs. 1A-2 and 1B-2). The profiles in the Fig. 1A (reduced proteins) and Fig. 1B (nonreduced proteins) were different.

1 To whom reprint requests should be addressed.
TABLE 1

<table>
<thead>
<tr>
<th>Virus</th>
<th>Neutralizing titer (50% plaque reduction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2D5</td>
</tr>
<tr>
<td>Vaccinia IHD-J</td>
<td>10^{1.4}</td>
</tr>
<tr>
<td>M1 mutant</td>
<td>&lt;10^{2}</td>
</tr>
<tr>
<td>M2 mutant</td>
<td>&lt;10^{2}</td>
</tr>
<tr>
<td>M4 mutant</td>
<td>&lt;10^{2}</td>
</tr>
<tr>
<td>Cowpox CPR-C1</td>
<td>10^{1.2}</td>
</tr>
<tr>
<td>Monkeypox Copenhagen</td>
<td>10^{1.7}</td>
</tr>
<tr>
<td>Mousepox Ishibashi-111</td>
<td>&lt;10^{2}</td>
</tr>
</tbody>
</table>

Note. Purified IMV samples were diluted with DMEM to give a titer of 2000 PFU/ml. Monoclonal antibodies and anti-vaccinia serum (1, 5, 13) were first diluted to 1/100 and then serially diluted by 3.2-fold. Virus solution (0.5 ml) was mixed with an equal volume of antibody and inoculated into Vero cell cultures (12-well plate, 50 μl/well) for plaque assay. Each value represents the mean of four assays.

due to a change of the protein configuration by reduction. The relationships are shown in the two-dimensional electrophoresis profiles presented in the literature (1, 6, 7). Overall, the viruses presented similar profiles except for a lack of VP32K in the D1 mutant virus (A-1) and slight differences in the cowpox and mousepox viruses. The silver-stained profiles indicated that VP23-29K was a minor component of the viruses, but the profiles stained with polyclonal anti-vaccinia mouse serum indicated that the nonreduced 23-kDa protein was a highly antigenic component common to all the tested viruses.

When the samples were stained with the 2D5mAb (Figs. 1A-3 and 1B-3), vaccinia viruses (IHD-J strain/WR strain/D1 mutant/vRB10 mutant), the CPR-C1 strain of cowpox virus, and the Copenhagen strain of monkeypox virus showed VP23-29K profiles similar to those of the reduced (29 kDa) and nonreduced forms (23, 21, and 19 kDa). In contrast, M2 mutant/M4 mutant/mousepox viruses did not react with the 2D5mAb. The nonreduced VP23-29K of the M1 mutant reacted with the 2D5mAb but the reduced form did not. All the virus reacted with the 8C2mAb at the nonreduced 23 kDa (Fig. 1B-4), but the 8C2mAb-reactive epitope disappeared after reduction. The 8C2mAb did not react with 21- and 19-kDa proteins. The results indicated that the 2D5mAb-resistant nature of the M2/M4/mousepox viruses coincided with the lack of reactivity against their VP23-29K. The VP23-29K of the M1 mutant had a variation different from those of the M2/M4/mousepox viruses.

A DNA fragment containing the promoter and the entire ORF sequence of A17L was cloned from genomic DNA of the IHD-J and the mutant viruses and then sequenced. The A17L genes of the M1 and M2 mutants and parental IHD-J strain virus were identical to that of the Copenhagen strain of vaccinia virus (8), except for G to A conversions at the nucleotides 120 and 360. The A17L of the M4 mutant contained a single point mutation (C to T) at the nucleotide 226 which would substitute serine for proline 67. To examine whether the corre-

FIG. 1. VP23-29K of orthopox viruses and 2D5mAb-resistant mutants. IHD-J strain vaccinia virus (lane 1), WR strain vaccinia virus (lane 2), D1 mutant (14) (lane 3), vRB10 mutant (15) (lane 4), M1 mutant (lane 5), M2 mutant (lane 6), M4 mutant (lane 7), CPR.C1 strain cowpox virus (lane 8), Ishibashi-111 strain mousepox virus (lane 9), and Copenhagen strain monkeypox virus (lane 10). The viruses were dissolved in reducing lysis buffer (A) or in nonreducing lysis buffer (B) and immunoblotted. (A-1 and B-1) Silver-stained profiles of viral proteins. (A-2 and B-2) Immunoblotting profiles with polyclonal anti-IHD-J strain vaccinia virus mouse serum. (A-3 and B-3) Immunoblotting profiles with 2D5mAb. (B-4) Immunoblotting profiles with 8C2mAb.
FIG. 2. Nucleotide sequences of A17L and L1R. Genomic DNA of CPR.C1 strain cowpox, Ishibashi-111 strain mousepox virus, and recHH1 was applied to PCR. Primers are indicated at both ends by boldface letters. The PCR products were cloned into pBluescriptII (KS* and SK*) and then sequenced. The sequences were lined up with those of the IHD-J strain vaccinia virus, as well as the M1, M2, and M4 mutants of which the entire genes were sequenced.

The corresponding protein of mousepox virus varies from the VP23-29K at amino acid 67 or nearby, the genetic region encoding amino acid residues 37–116 was amplified by PCR and sequenced (Fig. 2). The A17L equivalents of mousepox and cowpox viruses showed several different nucleotides, but the amino acid residues were not affected. Mousepox and cowpox viruses had amino acid sequences identical to that of the IHD-J strain virus concerning this region. The A17L genes of M1/M2/mousepox viruses were not involved in the antigenic variation found in their VP23-29K.

The resistance to 2D5mAb was considered to be encoded by a gene other than A17L. Genomic DNA of the M4 mutant virus was digested with HindIII, and the generated A to P fragments were isolated by agarose gel electrophoresis. Each of the DNA fragments was transfected into CV-1 cells infected with IHD-J strain virus (9), and the progeny was screened with the 2D5mAb. Resistance to the 2D5mAb was found in the progeny virus grown in cells transfected with the L fragment. The virus (recHH1) was plaque purified. Cultures transfected with the other DNA fragments did not produce 2D5mAb-resistant virus.

The HindIII L fragment of the M4 mutant DNA was cloned into a plasmid and cleaved with XbaI. The left half contained part of G9R, L1R, and a portion of L2R (nucleotide sequence of 79244–80965). The fragment was further cleaved with BamHI and the right end of HindIII/BamHI was digested for about 150 nucleotides. Transfection with this fragment (L1R of the M4 mutant) produced 2D5mAb-resistant virus.

The L1Rs of IHD-J, M1, and M4 viruses were sequenced. The M1 and M4 viruses contained the identical mutation of G to A at nucleotide 101 of the L1R ORF (80256). There was no other difference in the L1Rs. The mutation would substitute asparagine for aspartic acid 35. Part of the L1R ORF (80150–80378), corresponding to the coding region of the amino-terminal 74 amino acid residues, was amplified from genomic DNAs of the M2 mutant, mousepox, and cowpox viruses (Fig. 2). The M2 mutant and mousepox virus contained the same variation as that of M1/M4 mutants, whereas the amino acid se-
AID VY 7940 / 6a19$$$141 05-17-96 00:40:29 vira AP: Virology

IHD-J strain of vaccinia virus was replicated in the pres-

sensitive to the 2D5mAb. The substitution from aspara-

10.


6.

4.

5.

3.

2.

1.

sequence of cowpox virus was identical to that of the IHD-

J strain virus. Mousepox virus had an additional A to G 

gain or loss. The mutation at nucleotide 47 that would change the amino 

cidue of cowpox virus was identical to that of the IHD-

J strain virus. Mousepox virus had an additional A to G transition at nucleotide 47 that would change the amino acid residue from Glu-16 to Gly. The recHH1 contained the same mutation in the L1R as the M4 mutant virus, and its A17L coincided with that of the IHD-J strain virus. The recHH1 was a recombinant, not a spontaneous mu-

tation. The VP23-29K of the recombinant virus reacted with the 2D5mAb but not with the 2D5mAb. The IMV was purified. The IMV was dissociated in nonreducing lysis buffer and then precipitated with the 8C2mAb or 2D5mAb. The immune complex was collected on protein A–agarose beads and dissolved for electrophoresis (Fig. 3). When the immunoprecipitate by the 2D5mAb was dis-

sociated with nonreducing buffer, the 2D5mAb reacted with the 23- and 21-kDa proteins (Fig. 3B). The 8C2mAb reacted with its 23-kDa band, and distribution of tritium-labeled myristoylated protein coincided with the 23-kDa band. On the other hand, the 8C2mAb precipitated only the 23-kDa myristoylated protein, and it reacted with the 2D5mAb. Neither the 21- nor the 19-kDa protein was detectable in the 8C2mAb precipitate. The myristoylated protein in the precipitates migrated as a 29-kDa protein after reduction (Fig. 3A), and the reduction diminished antigenicity of the 21- and 19-kDa proteins. These results indicated that the gene encoding the VP23-29K was L1R, as reported by Wolfe et al. (3), and that the VP23-29K corresponded to the VP23-29K of IMV. The natures of the 21- and 19-kDa proteins were not clear.

The A17L product (VP21K) is assembled to the spheri-

lids, and then the virus was precipitated with the 8C2mAb but not with the 2D5mAb. The IMV was dissociated in nonreducing lysis buffer and then precipitated with the 8C2mAb or 2D5mAb. The immune complex was collected on protein A–agarose beads and dissolved for electrophoresis (Fig. 3). When the immunoprecipitate by the 2D5mAb was dis-

sociated with nonreducing buffer, the 2D5mAb reacted with the 23- and 21-kDa proteins (Fig. 3B). The 8C2mAb reacted with its 23-kDa band, and distribution of tritium-labeled myristoylated protein coincided with the 23-kDa band. On the other hand, the 8C2mAb precipitated only the 23-kDa myristoylated protein, and it reacted with the 2D5mAb. Neither the 21- nor the 19-kDa protein was detectable in the 8C2mAb precipitate. The myristoylated protein in the precipitates migrated as a 29-kDa protein after reduction (Fig. 3A), and the reduction diminished antigenicity of the 21- and 19-kDa proteins. These results indicated that the gene encoding the VP23-29K was L1R, as reported by Wolfe et al. (3), and that the VP23-29K corresponded to the VP23-29K of IMV. The natures of the 21- and 19-kDa proteins were not clear.

ACKNOWLEDGMENTS

We thank Dr. B. Moss for informing us about reactivity of the 2D5mAb. This work was supported by a grant-in-aid for scientific research from The Ministry of Education, Science and Culture, Japan.

REFERENCES