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Deletion of the Varicella-Zoster Virus Large Subunit of Ribonucleotide Reductase Impairs Growth of Virus In Vitro

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Cells infected with varicella-zoster virus (VZV) express a viral ribonucleotide reductase which is distinct from that present in uninfected cells. VZV open reading frames 18 and 19 (ORF18 and ORF19) are homologous to the herpes simplex virus type 1 genes encoding the small and large subunits of ribonucleotide reductase, respectively. We generated recombinant VZV by transfecting cultured cells with four overlapping cosmid DNAs. To construct a virus lacking ribonucleotide reductase, we deleted 97% of VZV ORF19 from one of the cosmids. Transfection of this cosmid with the other parental cosmids yielded a VZV mutant with a 2.3-kbp deletion confirmed by Southern blot analysis. Virus-specific ribonucleotide reductase activity was not detected in cells infected with VZV lacking ORF19. Infection of melanoma cells with ORF19-deleted VZV resulted in plaques smaller than those produced by infection with the parental VZV. The mutant virus also exhibited a growth rate slightly slower than that of the parental virus. Chemical inhibition of the VZV ribonucleotide reductase has been shown to potentiate the anti-VZV activity of acyclovir. Similarly, the concentration of acyclovir required to inhibit plaque formation by 50% was threefold lower for the VZV ribonucleotide reductase deletion mutants than for parental virus. We conclude that the VZV ribonucleotide reductase large subunit is not essential for virus infection in vitro; however, deletion of the gene impairs the growth of VZV in cell culture and renders the virus more susceptible to inhibition by acyclovir.

Varicella-zoster virus (VZV) is a neurotropic herpesvirus which causes chickenpox after primary infection and shingles upon reactivation of latent virus from dorsal root ganglia. The entire nucleotide sequence of VZV is known (6). Most VZV open reading frames (ORFs) have positional and sequence homologs in herpes simplex virus type 1 (HSV-1) (5). Since the functions of many HSV-1 gene products are known, the functions of their VZV counterparts may be predicted. Direct genetic studies of VZV, however, have lagged behind those of HSV-1, largely because of the inability to produce high-titer cell-free virus. A powerful method circumventing this problem in which infectious recombinant VZV is generated by transfecting a set of overlapping cosmids into cells permissive for viral replication was recently developed (3). Virus produced by using this approach should be isogenic, thus eliminating the need to plaque purify virus to isolate a given mutant.

Ribonucleotide reductase provides a key pathway for the formation of DNA precursors by catalyzing the reduction of ribonucleotides to the corresponding deoxyribonucleotides (for a review, see reference 27). Ribonucleotide reductase exists in all eukaryotic and prokaryotic organisms. Several viruses, including VZV and HSV-1, encode their own ribonucleotide reductases (4, 8, 12, 17, 20, 29, 34). Mammalian and bacterial ribonucleotide reductase as well as the enzymes expressed by the herpesviruses exist as complexes of two nonidentical subunits. The holoenzymes consists of two copies of each subunit, both of which are necessary for activity (27). The small and large subunits of HSV-1 ribonucleotide reductase were mapped to two contiguous ORFs in the unique long (U_L) region of the genome (22). Deletion of either of the HSV-1 ribonucleotide reductase subunits eliminates the enzyme activity (13, 26). Analysis of these deletion mutants revealed that HSV-1 ribonucleotide reductase activity is not essential for viral replication in cultured cells (14, 26). However, HSV-1 lacking this enzyme function is impaired for growth in cell culture, especially when the cells are grown in low serum concentrations or at high temperature (13, 14, 26).

Mammalian ribonucleotide reductase is under the strict negative allosteric control of dTTP and dATP (36). The activity of herpesvirus ribonucleotide reductase, however, is not down regulated by the accumulation of intracellular nucleotides (25). Therefore, during infection, viral ribonucleotide reductase activity results in an increase in the intracellular concentration of deoxynucleoside triphosphates (dNTPs). One implication of this is that the increased levels of dNTPs can compete with acyclovir triphosphate for binding to the viral DNA polymerase, thereby increasing the concentration of acyclovir needed to inhibit viral replication. Conversely, inhibitors of viral ribonucleotide reductase decrease the intracellular concentration of dGTP and increase the concentration of acyclovir triphosphate (31, 32), thus enhancing the activity of acyclovir against VZV and HSV-1 in vitro. Similarly, HSV-1 mutants lacking ribonucleotide reductase activity are more sensitive to acyclovir than is wild-type HSV-1 (2).

VZV ORF18 and ORF19 are homologous to the small and large subunits of HSV-1 ribonucleotide reductase, respectively (6). We confirmed that ORF19 encodes the large subunit of ribonucleotide reductase by demonstrating that cells infected with a VZV mutant deleted for ORF19 lack viral ribonucleotide reductase activity. Furthermore, we showed that VZV ribonucleotide reductase is not essential for viral replication in vitro. VZV deleted for ORF19, however, is impaired for growth in cell culture and exhibits increased sensitivity to acyclovir.

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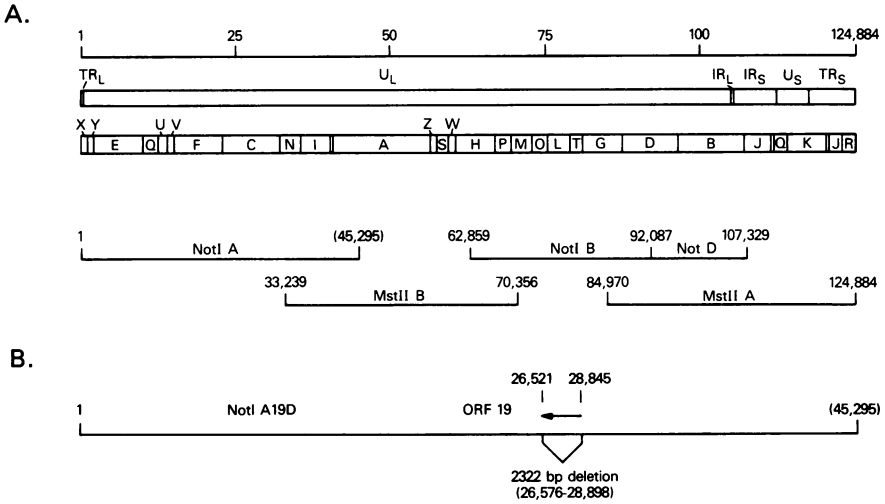

FIG. 1. Construction of recombinant VZV. (A) The prototype VZV genome (Dumas strain) is 124,884 bp in length (top line) and consists of terminal repeat (TR), unique long (U_L), internal repeat (IR), and unique short (U_S) DNA domains (second line). A *Bam*HI restriction endonuclease map is shown (third line). The four overlapping cosmids used to generate infectious virus are depicted (fourth and fifth lines). The cosmids are named according to the restriction fragments from which they were derived. The nucleotide coordinates correspond to the sequence of the prototype strain of VZV (6). The 3′ end of VZV *Not*I-A terminates within the R3 repeat region, resulting in deletion of the *Not*I site at the 3′ end of the VZV insert (indicated by parentheses) (3). (B) Cosmid *Not*I-A 19D contains a 2,322-bp deletion beginning 53 bp upstream of the ORF19 start codon and ending 55 bp upstream of the ORF19 stop codon.

MATERIALS AND METHODS

Cells and virus. MeWo cells, a human melanoma cell line, were maintained in minimum essential Eagle's medium (Quality Biologicals, Inc., Gaithersburg, Md.) supplemented with 10% fetal bovine serum (FBS). Recombinant VZV was propagated by inoculation of MeWo cells with virus-infected cells.

Cosmid DNAs. Four cosmids containing overlapping VZV DNA fragments from the Oka strain were used for MeWo cell transfections (Fig. 1) (3). The large subunit of ribonucleotide reductase is encoded by VZV ORF19, which lies between VZV nucleotides 28845 and 26521. To produce a cosmid deleted for VZV ORF19, the *Not*I-A cosmid was digested selectively with *Alu*I, using the RecA-assisted restriction endonuclease cleavage technique (10). Briefly, two 60-base oligonucleotides complementary to the VZV DNA strand encoding ORF19 and centered at the *Alu*I sites at VZV nucleotides 26576 and 28898 were synthesized. The 60-mers (90 ng of each) were incubated with the *Not*I-A cosmid DNA (1 μg) in the presence of 10 μg of *Escherichia coli* RecA protein for 10 min at 37°C. This results in a trimolecular complex in which the oligonucleotides are annealed to the complementary cosmid DNA sequences, thus protecting the two targeted *Alu*I sites from DNA-modifying enzymes. The remaining, unblocked *Alu*I sites in the *Not*I-A cosmid DNA were methylated with *Alu*I methylase in the presence of *S*-adenosylmethionine at 37°C for 20 min. After heat inactivation of the methylase and dissociation of the DNA-RecA complex, the DNA was digested with *Alu*I, which cuts at the two previously protected restriction sites, releasing a 2,322-bp DNA fragment containing most of ORF19. The cosmid DNA deleted for ORF19 was extracted with phenol, precipitated with ethanol, ligated with T4 DNA ligase, packaged into the heads of bacteriophage lambda particles (Stratagene, La Jolla, Calif.), and propagated in *E. coli* HB101. Plasmid pCMV62 contains the VZV ORF62 gene driven by the human cytomegalovirus immediate-early promoter (24).

Transfections. Sixty-millimeter-diameter dishes were seeded with 1.4×10^6 MeWo cells per dish 2 days before transfection. Transfections were performed by the calcium phosphate procedure (23). Each cosmid was linearized with *Not*I or *Mst*II, phenol extracted, and ethanol precipitated before transfection. Each transfection mixture included 0.5 μg of cosmid *Mst*II-A, 1 μg of each of the other three cosmids, 2 μg of sheared salmon sperm DNA, and 50 ng of plasmid pCMV62 (3). Five days after transfection, the cells were seeded into 75-cm^2 flasks and monitored for cytopathologic effects.

Southern blots. VZV DNA was purified from nucleocapsids of infected cells, cut with *Bam*HI or *Eco*RI, fractionated on a 0.6% agarose gel, and transferred to a nitrocellulose membrane. All four parental cosmid DNAs, spanning the entire VZV genome, were radiolabeled by random priming with [^{32}P]dCTP and hybridized to the immobilized VZV DNA.

PCR analysis. PCR was performed with the GeneAmp DNA amplification kit (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's protocol. The samples were incubated for 35 cycles consisting of a 30-s denaturation step at 97°C, a 2-min annealing step at 55°C, and a 3-min elongation step at 72°C. The template for cosmid DNA PCR was 5 to 10 ng of uncut cesium chloride-purified DNA. The template for PCR of virion DNA was a lysate of virus-infected cells. For each reaction, the two primers TGGACACTCCGGCCTGT AAA and TGCGATACAATTTAAGTCAA (VZV nucleotides 26448 to 26467 and 28987 to 29006, respectively) were used.

Ribonucleotide reductase assay. VZV-infected MeWo cells were harvested by scraping when they exhibited 3+ cytopathologic effects involving the entire monolayer. The cells were washed twice in phosphate-buffered saline and a final time in 50 mM Tris-HCl (pH 8.0) with 1 mM dithiothreitol. The swollen cell pellet was disrupted by sonication, and the crude extract was clarified by high-speed centrifugation at 14,000 × *g* for 5 min at 4°C. The protein concentration was determined by a colorimetric assay (Bio-Rad). One milligram of each extract was assayed for ribonucleotide reductase activity in a

200-μl reaction mixture containing [^{3}H]dCDP as previously described (18) except that 0.1 mM dATP was included in the reaction mixture (13) and ATP was omitted. The reaction mixture was incubated at 31°C for 1 h. dCMP was separated from CMP by thin-layer chromatography as described previously (29). The percentages of [^{3}H]CMP and [^{3}H]dCMP were quantified with a System 2000 image scanner (Bioscan, Inc., Washington, D.C.).

Immunoblot analysis. Aliquots of VZV-infected cell lysates from the same pools used for the ribonucleotide reductase assays were boiled in sample buffer containing sodium dodecyl sulfate (SDS) and 2-mercaptoethanol. The proteins were fractionated on an SDS–8% polyacrylamide gel and transferred to nitrocellulose. The blot was incubated for 2 h at room temperature with a 1:1,000 dilution of anti-VZV gpI mouse monoclonal antibody (Chemicon, Temecula, Calif.). After washing, goat anti-mouse antibody labeled with horseradish peroxidase was used to detect VZV gpI.

Growth characteristics and acyclovir sensitivity of recombinant VZV. The VZV titer was determined by inoculating MeWo cells with serial 10-fold dilutions of virus-infected cells (15). Seven days after infection, the cells were stained and the plaques were counted. The diameter of the stained plaques was measured with an inverted microscope calibrated to 0.1 mm at ×40 magnification. The statistical significance of the difference between parental and mutant VZV plaque size was determined by measuring 30 plaques in each group and applying Student's *t* test. VZV growth curves were generated by inoculating MeWo cells in 25-cm^2 flasks with infected cells containing 50 to 100 PFU of virus. Individual flasks were treated with trypsin, and the virus titer was determined on MeWo cells at days 1, 2, 3, 4, and 6 after infection.

The sensitivity of VZV to acyclovir was assayed by inoculating MeWo cells in 10-cm^2 wells with virus-infected cells containing 50 to 100 PFU of virus in the presence of acyclovir at 0, 0.5, 2.5, 10, and 25 μg/ml in the medium. Plaques were stained and counted 7 days after infection.

The sensitivity of VZV to the combination of acyclovir and the ribonucleotide reductase inhibitor 348U87 (kindly provided by Burroughs Wellcome, Inc., Research Triangle Park, N.C.) (30, 33) was also assessed. Compound 348U87 was reconstituted as a 3 mM solution in 0.1 N NaOH and used at a final concentration of 0.75 μM. MeWo cells were inoculated with VZV-infected cells in medium containing 348U87 reconstituted immediately prior to use and acyclovir at 0, 0.5, 2.5, 5, 10, and 25 μg/ml. On days 2, 4, and 6 after inoculation, the medium was replaced with medium containing acyclovir and newly dissolved 348U87 at the original concentrations. Plaques were stained and counted 7 days after infection.

RESULTS

Generation of VZV ORF19 deletion mutants. Infectious recombinant VZV can be produced by cotransfection of four overlapping cosmids which span the entire viral genome (Fig. 1A) (3). VZV ORF19, predicted to encode the large subunit of ribonucleotide reductase, is located in the *Not*I-A cosmid. (Fig. 1B). A 2,322-bp fragment of VZV DNA, extending from the *Alu*I site at VZV nucleotide 28898 to the *Alu*I site at nucleotide 26576, was deleted from the *Not*I-A cosmid. This deletion, which was confirmed by sequence analysis, begins 53 bp 5′ to the ORF19 start codon and ends 55 bp 5′ to the ORF19 stop codon, thereby eliminating 97% of the ORF19 coding sequence, including the start codon. VZV ORF20 is adjacent to ORF19; however, the deletion described above begins 107 bp downstream from the predicted polyadenylation site of ORF20 and thus is unlikely to interfere with expression of this gene product.

The deleted *Not*I-A cosmid (NotI-A 19D) was transfected with the three parental cosmids. Five days after transfection, the cells were passaged into 75-cm^2 flasks; 4 days later, changes typical of VZV infection appeared. Two independently derived VZV ORF19 deletion mutants were used in parallel for all subsequent experiments.

Southern blot analysis was performed to verify that the virus produced was deleted for VZV ORF19. Viral DNA from parental recombinant VZV (ROka) and from the two independently derived ORF19 deletion mutants (ROka 19D A and B) was digested with *Eco*RI or *Bam*HI and hybridized to the four labeled parental cosmid DNAs. The ROka DNA *Eco*RI H and *Bam*HI C fragments (Fig. 1) that contain ORF19 were replaced in ROka 19D with fragments that were 2.3 kb smaller, consistent with the expected size of the deletion in the VZV mutants (Fig. 2A and B). Longer exposure of the Southern blot indicated that the sizes of the smaller DNA bands were identical in VZV ROka and ROka 19D DNAs (16a).

The size and location of the deletion were also confirmed by PCR analysis (Fig. 2C). Amplification of VZV ROka DNA by PCR using primers bracketing ORF19 yielded a single DNA fragment of 2.6 kb, whereas PCR of ROka 19D DNA performed with the same primers resulted in a 0.23-kb DNA band.

VZV ribonucleotide reductase is dispensable for viral replication in cell culture. Viral ribonucleotide reductase activity was assayed by incubating lysates of VZV-infected cells with [^{3}H]CDP. To inhibit activity of the cellular enzyme, dATP was added to the reaction and ATP was omitted (13, 18). Viral ribonucleotide reductase activity was determined by assaying the conversion of [^{3}H]CDP to [^{3}H]dCDP. Lysates from cells infected with VZV ROka 19D exhibited a low level of ribonucleotide reductase activity (1.7% conversion of CDP to dCDP) similar to that observed in lysates from uninfected cells (1.6% conversion [Fig. 3A]). Lysates from cells infected with parental ROka, however, exhibited considerable ribonucleotide reductase activity (17% conversion [Fig. 3A]).

To confirm that the degree of VZV infection was similar in cell lysates used for the ribonucleotide reductase assays, immunoblot analysis was performed with aliquots of cell lysates from the same pools used for the enzyme assays. Cell lysates were run on a polyacrylamide gel, transferred to nitrocellulose, and incubated with a monoclonal antibody to VZV gpI. The amounts of gpI in the lysates from cells infected with each of the viruses were similar (Fig. 3B). Thus, the low level of viral ribonucleotide reductase activity in cells infected with ROka 19D was not due to the absence of VZV gene expression. These results demonstrate that ORF19 encodes a subunit of the VZV ribonucleotide reductase that is essential for enzymatic activity; however, VZV ribonucleotide reductase is dispensable for virus replication in vitro.

Absence of ribonucleotide reductase impairs the growth of VZV in cell culture. The plaque morphologies of cells infected with VZV ROka and ROka 19D were compared by inoculating MeWo cell monolayers with infected cells and growing the cells in the presence of 10% FBS for 7 days. VZV ROka 19D yielded distinctly smaller plaques than VZV ROka (Fig. 4). The mean size of plaques produced by infection of MeWo cells with VZV ROka was 1.1 ± 0.17 (standard deviation) mm, while the mean size of plaques produced by infection with ROka 19D was 0.34 ± 0.10 (standard deviation) mm ($P < 0.01$).

To further evaluate whether deletion of ribonucleotide reductase impairs the ability of VZV to grow in vitro, we measured virus production of ROka and ROka 19D during a

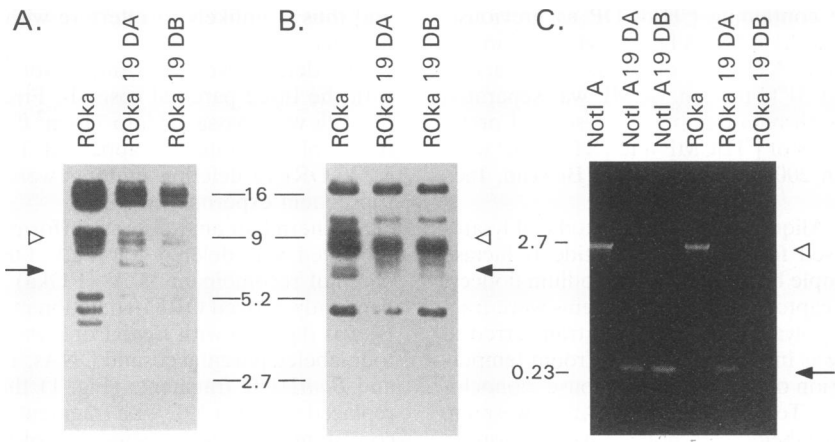

FIG. 2. Southern blot and PCR analysis of recombinant VZV DNA. (A and B) Virion DNA isolated from nucleocapsid preparations was digested with *Eco*RI (A) or *Bam*HI (B) and probed with all four radiolabeled cosmid DNAs. Digestion of VZV ROka DNA with *Eco*RI or *Bam*HI yields ORF19-containing fragments of 9.0 kb (A) or 9.6 kb (B) (arrowheads), respectively. Digestion of VZV ROka 19D DNA with *Eco*RI or *Bam*HI results in smaller (ORF19-deleted) fragments of 6.7 kb (A) and 7.3 kb (B) (arrows), respectively, consistent with the predicted 2,322-bp deletion. (C) Parental cosmid DNA (*Not*I-A), ORF19-deleted cosmid DNA (NotI-A 19D A and B), and lysates of MeWo cells infected with VZV ROka and VZV ROka 19D A and B were analyzed by PCR using primers bracketing the intended deletion. The resulting products were resolved on an ethidium bromide-stained agarose gel. PCR of *Not*I-A and ROka DNA yielded identical 2.6-kb fragments (open arrow), while PCR of *Not*I-A 19D A and B and ROka 19D A and B resulted in 0.23-kb DNA fragments, consistent with the 2,322-bp deletion (closed arrow). Numbers refer to size of DNAs in kilobase pairs.

6-day growth analysis. MeWo cells were inoculated with virus-infected cells in medium containing 10% FBS, and at various times after inoculation, the infected monolayers were harvested and the virus titer was determined. From 1 to 3 days after infection, the yields of PFU were similar for cells infected with ROka and ROka 19D. Thereafter, the number of PFU was lower in cells infected with ROka 19D than in cells infected with ROka (Fig. 5A). The results of both the plaque morphology and the growth curve analysis indicate that the absence of ribonucleotide reductase impairs the growth of VZV in vitro.

HSV-1 lacking ribonucleotide reductase is more severely

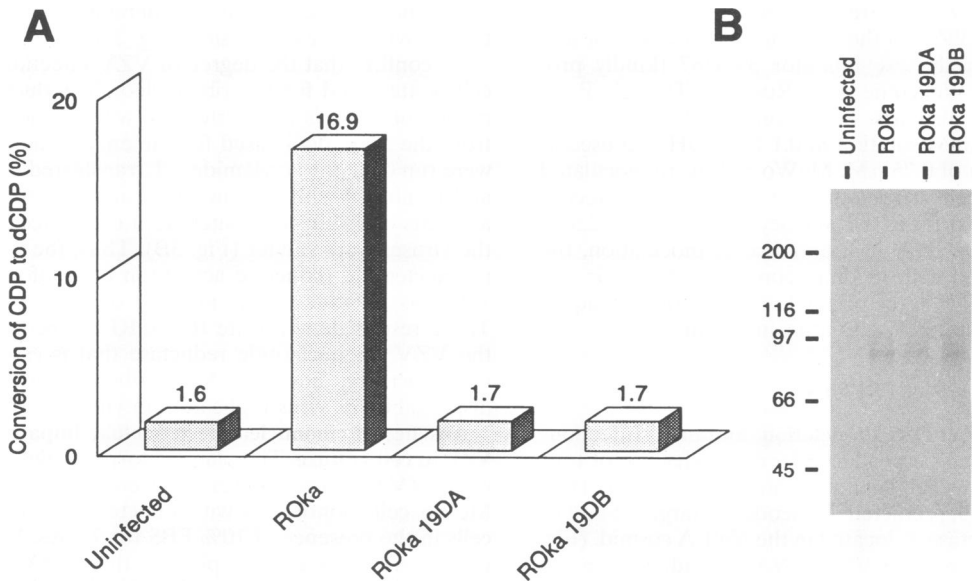

FIG. 3. Ribonucleotide reductase activity of cells infected with recombinant VZV. (A) Crude extracts containing 1 mg of protein from uninfected cells, cells infected with parental recombinant VZV (ROka), or cells infected with two independent VZV ORF19 deletion mutants (ROka 19D A and B) were assayed for ribonucleotide reductase activity by measuring the conversion of [^{3}H]CDP to [^{3}H]dCDP. The percentage of [^{3}H]CDP converted to [^{3}H]dCDP (counts in [^{3}H]dCMP spot on the chromatogram divided by the sum of the counts in the [^{3}H]dCMP and [^{3}H]CMP spots, all times 100) is indicated above the bars. (B) Immunoblot of protein extracts from the same pool of cells used in the ribonucleotide reductase activity assay. The blot was probed with a mouse monoclonal antibody to VZV gpI. VZV gpI is not present in the uninfected cell lysate, while the lysates from ROka- and ROka 19D-infected cells contain a family of bands ranging in size from 85 to 95 kDa corresponding to VZV gpI. Numbers refer to sizes of proteins in kilodaltons.

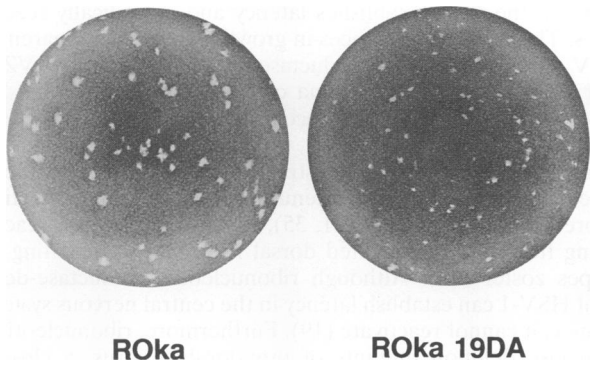

FIG. 4. Plaque size following infection of cells with VZV ROka or ROka 19D. MeWo cell monolayers were infected with cells containing similar titers of VZV ROka or ROka 19D and were incubated in medium containing 10% FBS for 7 days at 37°C prior to staining with crystal violet.

compromised in its ability to produce virus than is wild-type HSV-1 in serum-starved cells (13, 14). To determine whether VZV lacking ribonucleotide reductase behaves similarly, we repeated the virus yield studies by using MeWo cells grown in 0.5% FBS. Both VZV ROka and ROka 19D grew less well in serum-starved cells. However, VZV lacking ribonucleotide reductase was more impaired than the parental virus when the cells were grown in low serum (Fig. 5B).

Absence of ribonucleotide reductase increases sensitivity of VZV to acyclovir. Inhibitors of VZV ribonucleotide reductase potentiate the antiviral activity of acyclovir by increasing the intracellular ratio of acyclovir triphosphate to dGTP (31). To test whether deletion of the VZV ribonucleotide reductase increases the sensitivity of the virus to acyclovir, MeWo cells were inoculated with VZV-infected cells in the presence of various concentrations of acyclovir. Growth of VZV lacking ribonucleotide reductase was inhibited at lower concentrations of acyclovir than was growth of parental VZV (Fig. 6). The concentration of acyclovir required to inhibit plaque formation by 50% (IC_{50}) was 4.9 μg/ml for parental VZV, compared with 1.5 and 1.8 μg/ml for the two ribonucleotide reductase deletion mutants.

Chemical inhibition of ribonucleotide reductase potentiates the activity of acyclovir similarly to the effect seen with the VZV ribonucleotide reductase deletion mutants. Growth of the VZV ribonucleotide reductase deletion mutants in the presence of acyclovir was compared with the growth of the parental virus in the presence of a ribonucleotide reductase inhibitor (348U87) (30, 33). MeWo cells were inoculated with VZV-infected cells in the presence or absence of 0.75 μM inhibitor and various concentrations of acyclovir. The inhibitor had little or no effect on the growth of the VZV ribonucleotide reductase mutants in the presence of acyclovir. The inhibitor, however, reduced the growth of parental VZV in the presence of acyclovir to a level similar to that for the growth of the ribonucleotide reductase mutants in the presence of acyclovir without inhibitor (Fig. 7). The IC_{50} to acyclovir was 4.7 μg/ml for the parental virus without inhibitor and 0.65 to 1.75 μg/ml for the parental virus in the presence of inhibitor and for the ribonucleotide reductase mutants with or without inhibitor. These results suggest that there was complete or nearly complete inhibition of ribonucleotide reductase activity by the inhibitor at the concentration used. Use of higher concentrations of the inhibitor (>1 μM) resulted in extensive MeWo cell toxicity (16a).

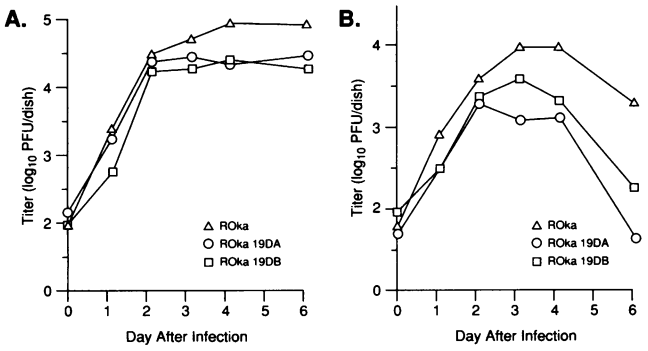

FIG. 5. Growth of VZV ROka and ROka 19D. MeWo cells were inoculated with VZV-infected cells and grown in either 10% (A) or 0.5% (B) FBS. Aliquots were harvested on days 1, 2, 3, 4, and 6 after infection, and titers were determined on MeWo cells. Day 0 value is the titer of virus in the VZV-infected cell inocula. The experiment was performed in duplicate, and the log_{10} of the mean number of plaques per dish at each time point is indicated.

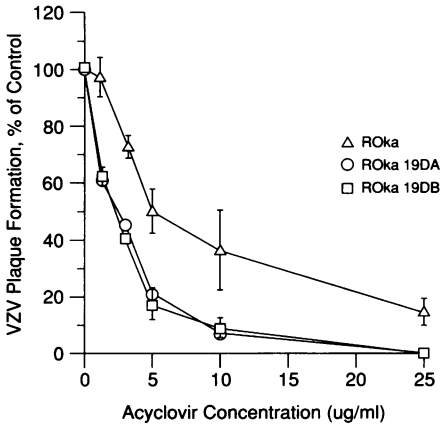

FIG. 6. Growth of VZV ROka and ROka 19D in the presence of acyclovir. MeWo cells were inoculated with VZV-infected cells in the presence of various concentrations of acyclovir, and plaques were counted 7 days after infection. VZV plaque formation is expressed as the percentage of the number of plaques seen in the presence of acyclovir divided by the number of plaques seen in the absence of acyclovir. The datum points shown represent the mean values from three independent experiments. The standard errors are indicated, and where overlap occurs, the largest standard error is shown.

DISCUSSION

We generated a VZV mutant deleted for the large subunit of ribonucleotide reductase and showed that this gene product is not essential for growth of the virus in cell culture. Construction of this mutant resulted in the deletion of 2,322 bp (2.2%) from the U_L region of the genome, including more than 97% of the ORF encoding the large subunit of ribonucleotide reductase. While deletions of similar size have been made in the U_L region of HSV-1 (13), the growth of the ribonucleotide reductase deletion mutant provides the first evidence that the VZV genome can tolerate an extensive deletion within its shorter U_L region.

Comparative analysis of the VZV and HSV-1 DNA sequences predicted that VZV ORF19 encodes the large subunit of ribonucleotide reductase. VZV ORF19 shares 44% amino

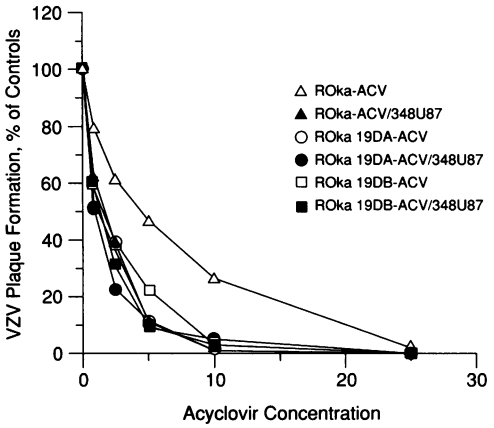

FIG. 7. Growth of VZV ROka and ROka 19D in the presence of acyclovir with or without the ribonucleotide reductase inhibitor 348U87. MeWo cells were inoculated with VZV-infected cells in the presence or absence of 0.75 μM inhibitor and various concentrations of acyclovir. Plaques were counted 7 days after infection. VZV plaque formation is expressed as the percentage of the number of plaques seen in the presence of acyclovir divided by the number of plaques seen in the absence of acyclovir. The datum points represent the mean values from four independent experiments.

acid identity with the carboxy two-thirds of the large subunit of the HSV-1 ribonucleotide reductase (6, 22). We confirmed this prediction experimentally by demonstrating viral ribonucleotide reductase activity after infection of cells with parental VZV but little or no enzyme activity following infection with VZV mutants lacking ORF19. Moreover, VZV, like HSV-1, does not require viral ribonucleotide reductase activity for growth in cell culture (13, 14, 26).

Deletion of the VZV large subunit of ribonucleotide reductase impairs the growth of virus in vitro, similar to the reduced growth seen in HSV-1 lacking ribonucleotide reductase activity (13, 14, 26). Infection of MeWo cells with VZV lacking ribonucleotide reductase activity yielded smaller plaques, and the virus grew at a rate slower than that seen following infection with parental recombinant VZV. Similar effects were noted with HSV-1 mutants lacking ribonucleotide reductase activity, and the differences between wild-type HSV-1 and ribonucleotide reductase deletion mutants were amplified when the infection was performed in serum-starved, nondividing cells (13, 14). While both of the VZV ribonucleotide reductase deletion mutants and the parental recombinant virus grew more slowly in low serum, growth of virus lacking ribonucleotide reductase was more impaired than was growth of parental virus. The difference in growth observed with VZV was less pronounced than has been reported for similar HSV-1 mutants. This apparent difference between the growth characteristics of ribonucleotide reductase-deficient VZV and HSV-1 might have resulted from differences in the cell lines used, incomplete growth arrest of the cells, or the method of infection (inoculation with VZV-infected cells) used in our study.

Alternative pathways for obtaining dNTPs, perhaps utilizing the cellular ribonucleotide reductase, may exist in dividing cells. Mammalian ribonucleotide reductase activity, however, is markedly diminished in cells arrested in the G_1 phase of the cell cycle that are not synthesizing DNA (9, 36). Thus, VZV ribonucleotide reductase may be more important in growth-arrested or terminally differentiated cells such as neural tissues in which the virus establishes latency and periodically reactivates. The modest differences in growth rate between parental VZV and ribonucleotide reductase deletion mutants of VZV that we observed in melanoma cells in culture might not be reflective of differences that occur during infection of differentiated cells in vivo.

In this regard, while the strain of VZV used for these experiments, strain Oka, is attenuated in humans and effective in preventing chickenpox (11, 35), it is still capable of reactivating from latently infected dorsal root ganglia, resulting in herpes zoster (16). Although ribonucleotide reductase-deficient HSV-1 can establish latency in the central nervous system of mice, it cannot reactivate (19). Furthermore, ribonucleotide reductase deletion mutants of pseudorabies virus, a closely related porcine alphaherpesvirus, are avirulent and elicit a protective immune response in pigs (7). These observations suggest that VZV strain Oka deleted for the large subunit of ribonucleotide reductase may ultimately prove to be a superior live virus vaccine strain.

VZV lacking ribonucleotide reductase exhibited an IC_{50} for acyclovir that was approximately one-third of the IC_{50} seen with parental virus. Since VZV ribonucleotide reductase activity is not allosterically inhibited by its metabolites, dNTPs accumulate to higher levels during infection and may compete more effectively with acyclovir triphosphate for binding to the viral DNA polymerase. Chemical inhibition of HSV-1 ribonucleotide reductase decreases the pool of intracellular dNTPs and increases the ratio of acyclovir triphosphate to its analog, dGTP, thereby enhancing the activity of acyclovir (31). Elimination of VZV ribonucleotide reductase activity probably potentiates the antiviral activity of acyclovir by the same mechanism. This view is supported by our observation that inhibition of VZV ribonucleotide reductase by compound 348U87 enhances the activity of acyclovir against VZV similarly to the effect seen with the ribonucleotide reductase mutants. The increased sensitivity to acyclovir of VZV strain Oka lacking ribonucleotide reductase activity may enhance the safety of the virus as a candidate vaccine.

VZV strain Oka is the only live DNA virus vaccine shown to be effective and safe in immunocompromised individuals (11). Thus, VZV Oka may serve as a useful vector for the expression of foreign viral gene products which may confer protective immunity to VZV as well as to other pathogens (21, 28). By demonstrating that the large subunit of VZV ribonucleotide reductase is not essential for replication in vitro and that deletion of this gene increases the sensitivity of the mutant VZV to acyclovir, we have identified a locus which may be suited to accept foreign genes for expression. Furthermore, since other alphaherpesviruses (e.g., HSV-1 and pseudorabies virus) lacking ribonucleotide reductase activity show reduced neurovirulence in animals (1, 7, 19, 37), the Oka strain of VZV lacking the large subunit of ribonucleotide reductase may be less neurovirulent in humans and therefore may be a safer live virus vaccine than the current Oka strain.

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