Guanidine-Resistant Poliovirus Mutants Produce Modified 37-Kilodalton Proteins

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Eighteen spontaneous, guanidine-resistant mutants of poliovirus were obtained by plaque selection. Isoelectric focusing demonstrated charge changes in a 37-kilodalton protein, pX, among three of the mutants. The precursor of pX, NCVP5b, also exhibited charge changes among the three mutants. pX of 12 mutants was also examined by peptide mapping with Staphylococcus aureus V8 protease. Nine of the mutants presented modified maps, and seven of these maps were identical. The demonstration of mutational changes in pX in 12 of 18 mutants suggests a role for this protein in determining the guanidine trait of poliovirus and corroborates studies with foot-and-mouth disease virus.

MATERIALS AND METHODS

Cells. HeLa-O cells from Flow Laboratories, Inc., and WISH (human amnion) cells from B. D. Korant at Du Pont Co. were grown in Corning flasks as monolayer cultures with Eagle minimal essential medium (MEM; 11) containing 7.5% fetal bovine serum, 50 μg of streptomycin per ml, and 50 U of penicillin per ml. Data obtained with WISH cells were identical to those obtained with HeLa cells; consequently, only studies with HeLa cells are included in this report.

Virus. Guanidine-resistant mutants were derived from the following strains of poliovirus: type 1 strains Mahoney (Mah), LSc, and Loddo; type 2 strain P712; and type 3 strain LH. All parent guanidine-sensitive viruses were plaque purified before isolation of gr mutants. Spontaneous g mutants were obtained via three to five plaque purifications with 100 μg of guanidine per ml in agar overlay medium. The Loddo prototype is guanidine dependent (g3) and g mutants were selected by plaquing in the absence of inhibitor.

Plaquing efficiencies. The effects of guanidine on growth of parent and mutant viruses were examined by assaying viruses with the plaque procedure in the absence and presence of 100 μg of guanidine per ml. Viruses exhibiting a significant (10-fold) reduction and increase in plaques in the presence of guanidine were designated g3 and g2, respectively. Viruses exhibiting moderate changes in titer in the presence of guanidine were considered g1 (Table 1).

Heat inactivation of virions. Virus samples were diluted 25-fold in Eagle MEM containing 2% newborn calf serum to concentrations of ca. 107 to 109 PFU/ml. One-milliliter samples were heated for 60 min in a 46 to 47°C water bath and quickly chilled in an ice bath, and viral infectivity was measured by the plaque procedure. Guanidine at 100 μg/ml was included in the agar overlay medium for the Loddo g3 virus.

Isoelectric focusing and two-dimensional analysis of poliovirus proteins. Cells (5 × 106) in 25-mm Corning flasks were

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Guanidine is a natural constituent of animal serum that acts as a protein denaturant at molar concentrations but at millimolar levels selectively blocks the growth of several togaviruses (12), several plant viruses (10, 29), and many picornaviruses, including poliovirus (3, 9, 18, 19, 21, 24). Although the exact mechanism of inhibition of viral growth is unknown, the major and most rapid effect of guanidine is blockade of viral RNA synthesis, particularly the production of single-stranded RNA (1–3, 16, 22). The specific site of inhibition appears to be the initiation step of RNA synthesis (3, 28). Other effects of guanidine, such as reduction of choline incorporation into membranes (23), interference with maturation of poliovirus (14), and prevention of capsid proteins of poliovirus from associating with smooth membranes (30), are probably secondary and due to limited synthesis of viral RNA.

Several genetic and biochemical studies with poliovirus have implicated capsid proteins or their precursor as determinants of guanidine sensitivity and resistance. Most guanidine resistance mutations map within or near the capsid region of the viral genome (7), and temperature-sensitive mutants with modified structural genes usually display a coordinate change in growth response to guanidine; temperature-sensitive mutants in nonstructural genes fail to manifest such a change (8). Guanidine-resistant (gr) mutants of type 2 poliovirus also differ from guanidine-sensitive (gs) virus in terms of stability of virions to high temperature, uncoating properties of virions, and chromatographic elution of capsid peptides (15).

Virus-induced polypeptides of guanidine-resistant mutants of foot-and-mouth disease virus have been examined by electrofocusing and trypptic peptide fingerprinting (25). In contrast to poliovirus, synthesis of an altered nonstructural polypeptide, p34, was noted with 5 of 10 mutants, which suggests that p34 is functionally involved in the antiviral action of guanidine. At present, p34 has no defined function and is probably the counterpart to poliovirus protein NCVPX (pX).

Saunders and King pointed out that serial passage of virus during selection procedures might have led to the isolation of double mutants in the poliovirus studies (25). Selection of g mutants requires plaque purification, which per se involves multiple virus growth cycles. Consequently, the potential for the selection of multiple mutants is an unavoidable hazard. Because of this problem we isolated a large number of g mutants of poliovirus and investigated in vivo proteins by electrofocusing and peptide mapping to identify a protein(s) that consistently changes with mutations of the guanidine marker. Our observations suggest that pX of poliovirus is the viral gene product responsible for the guanidine trait.

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infected with 50 to 75 PFU of virus per cell, and 40 to 60 μCi of [35S]methionine per ml was included in the medium (methionine-free) between 3 and 5.5 h postinfection. Cells were then washed three times with physiological saline and once with double-distilled water and lysed in 250 μl of a freshly prepared sample solution of 9.5 M urea, 4% Ampholine (pH 3.5 to 9.5), 1% 2-mercaptoethanol, and 1% Nonidet P-40. Cell debris was removed by centrifugation for 2 min at 12,000 × g in a microfuge, and samples were incubated at room temperature for 30 min with 100 μg of RNase.

First-dimension gels contained 5.25% acrylamide-bisacrylamide (30:0.8), 8 M urea, 2% Ampholine (pH 3.5 to 9.5), and 1% Nonidet P-40. Focusing was done with 0.75-mm vertical gels with an anode solution of 0.03 M H3PO4 (top of gel) and a cathode solution of 0.02 M NaOH. Each well was received 30 to 80 μl of sample (7.5 × 10−6 to 2 × 10−6 dpm), which was covered with 10 μl of an overlay solution of 4 M urea, 2% Ampholine (pH 3.5 to 9.5), 1% 2-mercaptoethanol, and 1% Nonidet P-40. Electrophoresis was performed at 100 V for 16 h, followed by 500 V for 1 h. The pH gradients of gels were measured with 1-cm2 gel sections after elution with 8 M urea in 10 mM KCl. Gels were occasionally fixed and stained with a solution of 25% isopropanol, 10% glacial acetic acid, 0.1% cupric acetate, and 0.1% Coomassie blue for 1 h, destained with 20% methanol and 10% acetic acid, dried, and covered with Kodak XAR-5 film at −80°C.

For the second-dimension gel, strips of 0.6 by 11 cm were excised and washed three times with a buffer of 0.2 M Tris-hydrochloride (pH 6.8) and 1% sodium dodecyl sulfate. Second-dimension separating gels were 1.2 mm by 14 cm with 14% acrylamide concentrations and 5% stacking gels (17). First-dimension gel strips were placed in contact with stacking gels and sealed with 1% agarose in a solution of 0.2 M Tris-hydrochloride (pH 6.8), 1% sodium dodecyl sulfate, and 0.01% bromphenol blue. Electrophoresis was generally performed for 4 h at 40 mA before fixing and impregnating gels with Enlightening or EnHance. After being dried, gels were covered with Kodak XAR-5 film at −80°C for 3 to 8 days. The following proteins were occasionally included as molecular-weight markers in both dimensions: myosin (200,000), β-galactosidase (117,000), phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,500).

**Peptide mapping of viral proteins.** To examine pX by proteolytic digestion, it was necessary to radioactively label viral proteins in vivo and perform electrophoresis with denaturing gels to separate other viral proteins from pX. To accomplish this, cells were infected as outlined above with 50 to 75 PFU of virus and incubated with 40 to 60 μCi of [35S]methionine per ml between 3 and 5.5 h postinfection. At the end of the pulse period, cells were washed with Hanks balanced salt solution, and extracts were prepared for polyacrylamide gel electrophoresis. After electrophoresis, gels were soaked in a staining solution of 39% methanol, 7.5% acetic acid, and 0.1% Coomassie blue for 15 min, followed by 10 min of destaining with a 7% solution of acetic acid. Brief staining and destaining were necessary to avoid acid hydrolysis of viral proteins. After being dried, gels were covered with Kodak XAR-5 X-ray film, which was developed 2 days later. Regions of gels containing pX were excised and soaked for 20 min in distilled water and for 30 min in a buffer of 0.5% sodium dodecyl sulfate, 8% glycerol,
80 mM Tris-hydrochloride (pH 6.8), 4% 2-β-mercaptoethanol, and 0.1% bromophenol blue. To obtain peptide maps of pX, gel slices of 1.2 mm in diameter were inserted into wells of 1.5-mm slab gels and covered with 15 μl of gel sample buffer containing 0.45 μg of *Staphylococcus aureus* V8 protease (4). Electrophoresis was performed with 40 mA until the dye band penetrated three-fourths of a 2-cm 5% stacking gel. Power was turned off for 45 min and then reinitiated at 40 mA until the dye band migrated 11 to 12 cm into the separating gel (12.5 to 18% linear). After electrophoresis, proteins were fixed for 1 h in a solution of 0.1% Coomassie blue, 25% isopropanol, 10% acetic acid, and 0.1% cupric acetate, destained with 7% acetic acid, treated with Enlightening, dried, and covered with Kodak XAR-5 X-ray film for 3 to 10 days. Radioactive, low-molecular-weight standards used with proteolytic digests were purchased from Bethesda Research Laboratories, Inc., Rockville, Md., and included the following: ovalbumin, 43,000; alpha-chymotrypsinogen, 25,700; β-lactoglobulin, 18,400; lysozyme, 14,500; cytochrome c, 12,300; bovine trypsin inhibitor, 6,200; and insulin, 3,000.

**Materials.** Eagle MEM, fetal bovine serum, calf serum, streptomycin, and penicillin were purchased from GIBCO Laboratories, Grand Island, N.Y. Acrylamide, bisacrylamide, TEMED (N,N',N',N'-tetramethylethylenediamine), sodium dodecyl sulfate, molecular weight markers, and urea were purchased from Bio-Rad Laboratories, Richmond, Calif.; electrophoresis grade agarose was obtained from Bethesda Research Laboratories. [35S]Methionine (900 to 1,200 Ci/mmol), En3Hance, and Enlightening were purchased from New England Nuclear Corp., Boston, Mass. Scinti-Verse was obtained from Fisher Scientific Co., Pittsburgh, Pa. Amphotilie was purchased from LKB Instruments, Inc., Rockville, Md., and electrophoretically pure RNase was purchased from Sigma Chemical Co., St. Louis, Mo. *S. aureus* V8 protease was purchased from Miles Laboratories, Inc., Elkhart, Ind.

**RESULTS**

**Isolation of gr mutants.** Because our paramount goal was to identify altered proteins among gr variants of poliovirus, it was necessary to take several precautions during the selection process. Our first concern was to avoid the selection of the same gr mutant from a single stock of g3 virus. To circumvent this problem, we initially plated g3 virus under agar overlay medium without guanidine and obtained stocks of g3 virus from different plaques in separate flasks. Spontaneous mutants that arose during growth of the various g3 viruses were then selected from plaques under agar overlay medium containing 100 μg of guanidine per ml. Hence, from Mah g31 we selected Mah g31, from Mah g32 we selected Mah g32, etc. Because the Loddo prototype is guanidine dependent, independent g3 variants were derived by plaque purification in the absence of inhibitor. Our second concern was to obtain g3 variants that were free of parent virus. To accomplish this, three to five plaque purifications were performed with guanidine. Excessive plaque isolations were avoided, and passages were minimized to prevent selection of double mutants. In this respect, virus was not serially grown between plaque selections. Virus that grew during each plaque purification step was directly plated for subsequent plaque selection, and only two to three passages were done after the last step to obtain working stocks. Titration of virus, with and without guanidine, between plaque selections showed a 100-fold purification of g3 variants during each of the first two plaque isolations.

The production of plaques in the absence and presence of guanidine was determined for parent and mutant viruses (Table 1). Plaque formation by g3 viruses was inhibited by ca. 4 to 5 log10. It should be noted that only one Mah g3 virus (i.e., Mah g31) is presented in Table 1. However, four separate isolates of Mah g3 virus have been studied, and all were identical in plaquing efficiencies with guanidine, thermal sensitivity of virions, and pI measurements of proteins produced in vivo (data not shown).

Plaquing efficiencies of guanidine mutants in the presence of guanidine were variable. Of the 18 g3 mutants, 13 produced higher numbers of plaques than g3, incorporated into the agar overlay medium, demonstrating a moderate guanidine dependence. However, the g3 designation was maintained in preference to g3 (dependent) because enhancement of growth was moderate compared with that of the Loddo g3 prototype.

**Temperature inactivation of virions.** Previous investigations with guanidine mutants of poliovirus showed changes in temperature sensitivity of virions compared with parent virions (15). These modifications in thermal inactivation were shown to arise from mutations in the capsid locus. Heat inactivation of g3 mutants was also performed in the present study as a preliminary screening procedure for alterations in coat proteins. Samples of virus were diluted 25-fold with MEM containing 3% newborn calf serum and incubated in a water bath at 46 to 47°C for 60 min; infectivity was measured by plaque assay. A control virus with a mutation in VP2, Mah g3 was included as a reference. The mutational change(s) in this mutant was not detectable by electrofocusing, but a modification in VP2 was apparent when proteolytic digests with V8 protease or elastase were analyzed (data not shown). This virus is most likely a multiple mutant that was plaque purified after several passages with guanidine in the medium.

The overall observation from heat inactivation studies is that g3 mutants exhibited thermal sensitivities that are characteristic of the parent viruses from which they were derived. Over a 60-min period at 46 to 47°C, infectivity was decreased by 3.0 ± 0.3 log10 for Mah, LSc, and LFI virions. Mutants demonstrating a degree of guanidine dependence such as Mah g31 were equally sensitive to heat when assays were performed with or without guanidine (data not shown). Loddo virions were more resistant and P712 virions were more sensitive to heat than other strains of virus were, but mutation of the guanidine marker had no effect on thermal sensitivities. In addition to the data in Table 1, several g3 variants and their respective parent viruses were examined after exposure to heat for 15, 30, 45, and 60 min to detect slight nuances in activation kinetics, but none were observed (data not shown). In summary, none of the mutants that were carefully selected exhibited changes in thermal sensitivities. The reference virus, Mah g3, which showed a marked increase in temperature lability, underwent five serial passages with guanidine and two passages without guanidine before plaque purification. Serial passage might have led to the derivation of a multiple mutant.

**Isoelectric focusing and two-dimensional gels.** Theoretically, a charge modification in a designated protein is anticipated in approximately one-third of missense mutations (26). If missense mutations are responsible for the majority of g3 mutants, then it should be possible to identify altered viral proteins in extracts of cells infected with these mutants. In our investigations, HeLa cells were infected with mutants or parent virus at 50 to 75 PFU per cell, and [35S]Methionine at 40 to 60 μCi/ml was included in the medium from 3 to 5.5 h...
postinfection, a time period when synthesis of cellular proteins is severely restricted. In vivo viral proteins were then subjected to two-dimensional gel analysis. Before the data are discussed, several features of two-dimensional gels and identification of viral proteins warrant discussion.

It is mandatory to identify viral proteins against a small but observable background of cellular proteins. Three sets of data enabled us to accomplish this. First, the pI values of structural proteins of Mahoney virus were previously determined by others, and this established a reference for our experiments with the Mahoney prototype (13). Second, we showed that capsid proteins were absent from two-dimensional gels containing extracts from cells infected with defective interfering (DI) particles of Mahoney virus (compare Fig. 1 and Fig. 3A). DI particles are deletion mutants in the capsid locus and do not produce capsid proteins during infection (5, 6, 20, 27). The data in Fig. 1 were obtained with g<sup>+</sup> and g<sup>−</sup> derivatives of DI particles (27). Third, molecular weight markers were incorporated into both dimensions of the experiment so that we could evaluate molecular weights of virus-specific peptides. This combination of controls enabled us to identify the majority of structural and non-structural virus-specific proteins with confidence.

All experiments were done with Ampholines of pH 3.5 to 9.5, but narrow-range Ampholines of pH 5 to 7 and 6 to 8 were also tested with half of our mutants. Narrow-range Ampholines did not significantly enhance the sensitivity of the experiments or unmask charge changes that might have been missed by our standard pH 3.5 to 9.5 gels. VP3 and, to a lesser extent, VP1 had a tendency to precipitate and smear

FIG. 1. Two-dimensional polyacrylamide gels of in vivo proteins produced by DI particles. (A) Cells infected with 50 PFU equivalents of a g<sup>+</sup> DI particle and exposed to [35S]methionine from 3 to 5.5 h postinfection; (B) study identical to that in (A) except for infection with a g<sup>−</sup> variant of DI particles.

FIG. 2. Two-dimensional polyacrylamide gels of in vivo Loddo g<sup>+</sup> and g<sup>−</sup> proteins. (A) Extracts from cells infected with Loddo g<sup>+</sup> virus; (B) coelectrophoresis of extracts from cells infected with Loddo g<sup>+</sup> and Loddo g<sup>−</sup>2 viruses.
mutants of viruses was performed, and the consistent differences between g3 and g2 viruses were tabulated. The figures represent data obtained with only one g3 virus, i.e., g3, but pXs of two other g2 isolates were examined and displayed peptide maps identical to that obtained with g3 virus.

Peptide maps of pX of g1 virus were the most diversified at the point of application, i.e., the tops of the gels. This feature was also noted by others (13). Consequently, we performed a minimum of five experiments with each virus to ensure that our observations were not procedural artifacts.

Our results (Fig. 1 to 3), summarized in Table 1, show that 3 of 18 g2 mutants displayed changes in pI values of NCVP5b and NCVPX (hereafter the prefix p will replace NCVP). The Loddo g2 mutant also manifested a charge modification in p3b (Fig. 2). During poliovirus growth, p3b is processed proteolytically into p5b, which is subsequently converted to pX. We have occasionally detected changes in p3b of Mah g3 and g4 but are not concerned by the failure to consistently find this. Our pulse procedures are conducted in the later stages of infection when p3b is converted to p5b. The important observation is that pX, which represents ca. 50% of the carboxyl region of p3b, contains pI changes.

Two-dimensional gels, from which the above information was derived for Loddo g2, Mah g3, and g4, are presented in Fig. 2 and 3 as representatives of several studies. To accurately assess mutants, extracts from cells infected with parent viruses were coelectrophoresed with extracts from cells infected with mutants. A charge modification in a mutant protein should then provide doublet spots on films as exemplified in Fig. 2B, 3B, and 3C. From relative positions of proteins, it was possible to categorize the pI change; one was basic and two were acidic. Of the three mutants illustrated in the figures, all contained mutations in p5b and pX, and Loddo g2 also contained a demonstrable change in p3b. The data in Fig. 3 were obtained by coelectrophoresis of proteins of Mah g2 virus with proteins of g3 and g4 mutants. The differences noted are not due to g1 virus. We have performed identical studies with proteins of the original parent Mah g3 virus and four other g2 isolates, with identical results.

Peptide mapping. Isoelectric focusing demonstrated charge alterations among 17% of the mutants we examined. This percentage is only half of that theoretically predicted for missense mutations (26). The low frequency of charge modifications might arise from interference with the growth of mutants by parent virus. Alternatively, a preponderance of missense mutations without charge modifications might be prevalent in the pX locus. We extended our investigations by obtaining peptide maps of pX of Mah g2 variants with S. aureus V8 protease. Peptide profiles of digested proteins exhibited differences among 9 of the 12 mutants. Part of the data is presented in Fig. 4 and 5, and the composite of all studies is summarized in Table 2. Faint bands of radioactive peptides were occasionally detected but were not considered in the final analysis. A minimum of four separate experiments were performed, and the consistent differences between g3 and g2 viruses were tabulated. The figures represent data obtained with only one g3 virus, i.e., g3, but pXs of two other g2 isolates were examined and displayed peptide maps identical to that obtained with g3 virus.

Peptide maps of pX of g1 virus were the most diversified.

FIG. 3. Two-dimensional polyacrylamide gels of in vivo viral proteins. (A) Mah g1; (B) coelectrophoresis of extracts from cells infected with Mah g1 and Mah g3; (C) coelectrophoresis of extracts from cells infected with Mah g1 and Mah g4. X, pX (i.e., NCVPX).
(Fig. 4). At least five peptides were detected with modified molecular weights compared with \( g^s \) virus, and three peptides were absent. Of the remaining mutants, eight also consistently showed a decrease of 300 to 400 in molecular weight of two peptides of 15,500 and 13,000. Although the changes are small, they were reproducible in four separate experiments. V8 protease also generated a peptide of 9 kilodaltons among these eight mutants but not among the three \( g^s \) isolates we examined. The low level of radioactivity in the \( g^7 \) sample of Fig. 5 precludes the detection of 15.5- and 9-kilodalton peptides, but in other studies these proteins were clearly discernible. Slight degradation of pX in the untreated control is not due to contaminating proteins but occurs during acetic acid fixation of the primary gel from which sections of gels were excised for mapping.

FIG. 5. Peptide maps of pX. The experiment was performed as described in the legend to Fig. 4 with a different series of mutants. Kd, Kilodaltons.

DISCUSSION

Data with poliovirus and foot-and-mouth disease virus suggest a role for capsid proteins and nonstructural proteins, respectively, for the determination of guanidine sensitivity of viral growth. Conflicting data are not surprising because of methods employed for the isolation of \( g^f \) variants. Selection of mutants by plaque procedures necessitates multiple virus growth cycles, and multiple mutants are an unavoidable risk. Since the capsid locus encompasses ca. 40% of the viral genome, mutations in this region might occur with regularity and complicate genetic experiments (7, 8).

In the present study, we selected 18 \( g^s \) mutants of poliovirus that arose spontaneously among separate stocks of \( g^s \) virus. Seventeen percent contained charge changes in pX and its precursor, NCVP5b. Twice that level of charge modifications are expected for missense mutations (26). The low frequency of such changes could arise from frameshift mutations, interference phenomena, or a predominance of amino acid substitutions involving neutral amino acids. The possibility that silent mutations occurred in regions of the genome coding for proteins other than pX cannot be excluded by isoelectric focusing data alone.

Peptide maps of pX that were derived with V8 protease consistently showed differences between \( g^s \) parent virus and nine \( g^s \) mutants. Although data are not presented here, we also analyzed pX of \( g^s \)1 virus and \( g^s \)7 through \( g^s \)12 mutants with elastase and found differences in peptide maps between the \( g^s \) virus and all six mutants. Because partial digests were obtained, the presence of several altered peptides among \( g^s \) variants does not imply multiple mutations. The minor peptides generated by V8 protease are probably derived from larger precursors, and a single amino acid change could conceivably cause modification among several peptides. We also analyzed p2 (NCVP2) and p4 (NCVP4) of five mutants with V8 protease but found no changes, even among minor peptides that were produced by enzymatic digestion (data not shown). The summary of all of our experiments shows modifications in pX among 75% of our \( g^s \) mutants and is in agreement with published data concerning foot-and-mouth disease virus (25). Whether the mutations are clustered in one region of the pX locus or scattered broadly over the locus cannot be determined without nucleotide sequence analysis of the \( g^s \) variants.

Because guanidine appears to interfere with the initiation step of RNA synthesis (3, 28), a role for pX during RNA synthesis is implied. pX might be critical for membrane association of P0b, a polymerase precursor, and might determine translocation from rough to smooth endoplasmic reticulum during RNA synthesis. The functional turnover of viral polymerase requires continuous replenishment during RNA synthesis, and interference with the transport of precursor proteins would restrict the initiation of synthesis of

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<th>Virus</th>
<th>Change(s) noted among peptides produced with S. aureus V8 protease*</th>
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* +, Change in molecular weight of peptide; + (M), peptide missing; + (N), new peptide; -, no change.

b Molecular weight in thousands.
viral RNA. A recent report with human rhinovirus type 2 also suggests a role for pX and a host factor(s) during synthesis of viral RNA (31). Host range mutants of human rhinovirus type 2 capable of growing in mouse L-cells were selected after multiple passages in L-cells. pXs of these variants were 45 versus 43 kilodaltons for parent virus, and no detectable changes occurred among structural peptides.

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LITERATURE CITED