Three Amino Acid Substitutions in the L Protein of the Human Parainfluenza Virus Type 3 \( cp45 \) Live Attenuated Vaccine Candidate Contribute to Its Temperature-Sensitive and Attenuation Phenotypes

MARIO H. SKIADOPoulos,\( ^1* \) ANNA P. DURBIN,\( ^1 \) JOANNE M. TATEM,\( ^2 \) SHIN-LU WU,\( ^2 \) MARIBEL PASCHALIS,\( ^2 \) TAO TAO,\( ^1 \) PETER L. COLLINS,\( ^1 \) AND BRIAN R. MURPHY\( ^1 \)

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland 20892,\( ^2 \) and Wyeth-Lederle Vaccines and Pediatrics, Pearl River, New York 10965\( ^2 \)

Received 10 October 1997/Accepted 24 November 1997

Human parainfluenza virus type 3 (PIV3), a member of the genus Paramyxovirus of the family Paramyxoviridae, has a single-stranded, negative-sense RNA genome that is 15,462 nucleotides (nt) in length. PIV3 is a major cause of serious lower respiratory illness requiring hospitalization of infants and young children (4). A vaccine is needed to prevent the severe disease caused by this virus, and two live attenuated candidate PIV3 vaccines are currently being evaluated in humans (21, 24, 32) and since preliminary data suggested that the L gene of PIV3 candidate vaccines such as \( cp45 \) possesses multiple point mutations in coding and noncoding regions of the genome, including three point mutations in the L polymerase gene that each encode an amino acid substitution (34).

Previously, we recovered recombinant PIV3 from a full-length antigenomic cDNA clone of wt PIV3 (JS strain), the parent of \( cp45 \), and demonstrated that the recovered virus was not temperature sensitive and replicated to a level in the respiratory tract of rodents comparable to that of the biologically derived wt (JS strain) virus (9). This meant that it was now possible to systematically examine the genetic basis of the \( att \) phenotype of PIV3 candidate vaccines such as \( cp45 \). Since the polymerase genes are the sites of many \( att \) and \( ts \) mutations for influenza virus and respiratory syncytial virus (RSV) (8, 11, 20, 24, 32) and since preliminary data suggested that the L gene of \( cp45 \) possesses a \( ts \) mutation (30), we initiated our studies to examine the genetic basis of attenuation of \( cp45 \) by introducing the mutations yielding the seven possible combinations of the three amino acid substitutions present in the L gene of \( cp45 \) into the cDNA clone of its wt (JS strain) parent. Seven recombinant viruses (three single, three double, and one triple mutant) were isolated and analyzed for their \( ts \) and \( att \) phenotypes. Analysis of these mutants indicated that each of the three mutations in the L protein is a major separate contributor to the \( ts \) and \( att \) phenotypes of this promising vaccine candidate. Furthermore, this study illustrates the usefulness of
The various mutant L plasmids supported marker gene expression at 75 to 106% consisting of plasmid-encoded minigenome RNA and N, P, and L proteins (10). was determined and was found to differ from the wild-type sequence only in the otide sequence of the 2.7-kb nt 11317 to 13733) fragment containing all seven point mutations in pUCL(N-S)

The newly developed reverse-genetics systems for characterizing and manipulating a nonsegmented negative-strand virus.

MATERIALS AND METHODS

Viruses and cells. The PIV3 wt (JS strain) and cp45 viruses were grown in simian LLC-MK2 cells as described previously (12). The T7F3 recombinant vaccinia virus (12) and the modified vaccinia virus Ankara (MVA-T7) (36), which each express the T7 polymerase, were kindly provided by Linda Wyatt and Bernard Moss. HeP-2 (ATCC CCL 23) and LLC-MK2 (ATCC CCL 7.1) cells were maintained in OptiMEM (Life Technologies, Gaithersburg, Md.) supplemented with 2% fetal bovine serum (FBS) and gentamicin sulfate (50 μg/ml). L-132 cells (ATCC CCL 5) were grown in Earle’s minimal essential medium (Life Technologies) supplemented with 10% FBS, 2 mM glutamine, 20 mM HEPES, 1 mM nonessential amino acids, and 100 U of streptomycin-neomycin/ml.

Construction of point mutations in the L gene of PIV3, pUC19 was modified to accept a fragment of the wt (JS strain) PIV3 L gene to introduce point mutations into the L gene by site-directed mutagenesis. First, a unique restriction site of pUC19 to create pUC19(N). We previously described the construction and functional testing of pTM(L), which includes the positions where the three coding substitutions at L protein positions 942, 992, and 1558, individually and in combination, and (ii) ablating one specific naturally occurring restriction enzyme recognition sites had been ablated during insertion of the three cp45 amino acid substitution mutations in L (see Table 2).

Efficiency of plaque formation at permissive and restrictive temperatures of rPIV3 bearing one, two, or three cp45 L protein amino acid substitutions. The levels of temperature sensitivity of plaque formation in vitro of control and recombinant viruses were determined at 32, 37, 38, 39, 40, and 41°C. The T7 polymerase and wt rPIV3 (JS strain), PIV3 +/− cp45 reference stocks were used as controls.

Hamster studies. Four- to 16-week-old golden Syrian hamsters in groups of six were inoculated intranasally with 0.1 ml of OptiMEM containing 10^6 PFU of wt rPIV3 (JS strain), PIV3 cp45, or one of the rPIV3s containing one or more cp45 L protein amino acid substitution(s). On day 4 postinfection, the hamsters were sacrificed, the lungs and nasal turbinates were harvested, and the virus was quantified as described previously (9). The mean log_{10} 50% tissue culture infectious dose per gram was calculated for each group of six hamsters.

RESULTS

Introduction of the PIV3 cp45 L protein amino acid substitution mutations into wt rPIV3 (JS strain). Mutations yielding the amino acid substitution result in the L protein amino acid substitution(s). On day 4 postinfection, the hamsters were sacrificed, the lungs and nasal turbinates were harvested, and the virus was quantified as described previously (9). The mean log_{10} 50% tissue culture infectious dose per gram was calculated for each group of six hamsters.
TABLE 1. Nucleotide changes introduced into rPIV3 that yield cp45 L protein amino acid substitutions and, as markers, ablate naturally occurring restriction enzyme sites

<table>
<thead>
<tr>
<th>rPIV3 designation</th>
<th>Amino acid substitution (wt to cp45)</th>
<th>Nucleotide sequencea</th>
<th>Restriction enzyme site ablated</th>
</tr>
</thead>
<tbody>
<tr>
<td>r942</td>
<td>Tyr-942 to His</td>
<td>11468-TTAACCATGCCCCAT</td>
<td>EaeI</td>
</tr>
<tr>
<td>r992</td>
<td>Leu-992 to Phe</td>
<td>11618-TCATTGCCCCGC</td>
<td>BglI</td>
</tr>
<tr>
<td>r1558</td>
<td>Thr-1558 to Ile</td>
<td>13307-TGAGCACICAATCTG</td>
<td>AvaII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13307-TGAGCAATATATCG</td>
<td></td>
</tr>
</tbody>
</table>

a The nucleotide sequence around each of the three mutated regions is shown. The first nucleotide in each provided sequence is numbered according to its position in the wt sequence, which were ablated to mark the mutation, are in italics. The nucleotides that were mutated to produce an amino acid substitution or remove a restriction enzyme site are underlined.

mutation in recovered rPIV3 (Table 1). In the engineered virus r1558, the coding change resulting in a substitution at amino acid (aa) 1558 was designed to contain two nucleotide changes, compared to the 1-nt substitution in cp45, to reduce the chance of reversion at this site during in vitro or in vivo replication (Table 1). It was not possible to do the same for the recombinant viruses with mutations coding for changes at positions 942 and 992 (i.e., r942 and r992). Seven rPIV3s bearing one, two, or all three of the amino acid substitutions were recovered in tissue culture by transfection of each antigenomic cDNA together with the pTM(N), pTM(P), and pTM(L) support plasmids and coinfection with the vaccinia virus MVA-T7 recombinant. Each rPIV3 possessed the MAb resistance marker that had been deliberately introduced into the HN gene by engineering the antigenic cDNA (9) (data not shown). The rPIV3s were biologically cloned by two or three cycles of plaque-to-plaque passage to ensure that each virus preparation was genetically homogeneous. This precaution was taken because vaccinia virus can mediate recombination between the antigenic cDNA and the support plasmids, as has been demonstrated in a comparable system (14).

We first sought to confirm that each of the seven rPIV3s contained the engineered mutation(s) in the L gene. vRNA was purified from precipitated virions, copied into cDNA, and amplified by RT-PCR. Control reactions showed that the RT step was required for generation of RT-PCR products, indicating that an RNA template rather than contaminating cDNA was required for the generation of the RT-PCR product (Fig. 2). The RT-PCR products were subjected to digestion with the three restriction enzymes whose recognition sequences had been ablated as markers for the inserted coding changes (Fig. 3). As expected, the RT-PCR product of wt rPIV3 was cleaved the appropriate number of times by each of the three enzymes (Fig. 3, lanes 8), whereas r942/992/1558 (Fig. 3, lanes 7) lacked each of the three sites ablated during creation of the individual cp45 coding changes. Each of the other rPIV3s lacked the appropriate restriction site(s), indicating the presence of the introduced mutation(s).

Efficiency of plaque formation in LLC-MK2 cells of rPIV3s bearing the cp45 L gene mutations at permissive and restrictive temperatures. The seven rPIV3s bearing the various combinations of cp45 L protein amino acid substitutions were assayed for their ability to form plaques on LLC-MK2 monolayers at various permissive and restrictive temperatures (Table 2). As shown in Table 2, each rPIV3 bearing a cp45 L protein amino acid substitution was temperature sensitive, whereas the wt rPIV3 parent (JS strain) was not restricted in plaque formation at any temperature tested. The shutoff temperature of plaque formation for r942 and r992 was 40°C. At 40°C, r942 manifested a 300-fold reduction of plaque formation and formed plaques that were small in size, indicating that its replication was reduced at this restrictive temperature; at 41°C, it failed to form plaques. r992 demonstrated greatly reduced plaque formation (more than a 10²-fold reduction) at 40°C. The shutoff temperature of plaque formation for r1558 was 39°C. These results indicate that each of the three cp45 amino acid substitution mutations individually specifies the ts phenotype, although the mutation yielding the substitution at aa 942 specifies the lowest level of temperature sensitivity. The double-mutant virus r942/1558 had a shutoff temperature of 39°C, while that of r942/992 and cp45 was 38°C. Thus, for these two double mutants, a combination of cp45 amino acid substi-
tutions provides a greater degree of temperature sensitivity than individual amino acid substitutions do. The third double mutant, r992/1558, exhibited only a 250-fold reduction in titer at 40°C and thus is less temperature sensitive than the r992 and r1558 mutants bearing individual mutations. This shows that combining these two particular cp45 mutations reduces rather than increases the level of temperature sensitivity. The finding that the double-mutant virus r942/992 is more temperature sensitive than r942/992/1558 is a second indication that certain combinations of mutations result in a reduced rather than increased level of temperature sensitivity. These observations suggest that the temperature sensitivity specified by the L gene of cp45 is a function of an interaction between the three amino acid substitution mutations rather than simply the sum of their individual effects. Also, the temperature sensitivity of r942/992/1558 is comparable to that of cp45, suggesting that the three mutations at positions 942, 992, and 1558 are major determinants of its temperature sensitivity.

The cold-adapted (ca) phenotype is manifested by the ability of a mutant virus to replicate more efficiently than wt virus at the suboptimal temperature of 20°C (1). Each of the seven rPIV3s with the L gene mutations failed to specify the ca phenotype, indicating that the cp45 mutation(s) responsible for the ca phenotype lies outside of the L gene sequence (data not shown).

Growth in hamsters. Groups of six hamsters were inoculated intranasally with wt rPIV3 (JS strain), biologically derived cp45, or an rPIV3 containing one or more cp45 L protein amino acid substitutions, and the level of virus replication in the lungs and nasal turbinates was determined 4 days later. The peak virus titer of PIV3- and cp45-infected hamsters was previously demonstrated to occur on day 4 postinfection (6). Each of the rPIV3s bearing a single amino acid substitution was restricted in replication in the upper and lower respiratory tracts (Table 3). Although r942, the least temperature-sensitive virus, was only marginally suppressed in replication, the mutation yielding a substitution at aa 942 clearly contributed to attenuation when present in a double- or triple-mutant recombinant. These data indicate that each of the three amino acid substitutions in the L protein of cp45 contributes to the att phenotype. The triple mutant r942/992/1558 was as restricted in replication in the upper and the lower respiratory tracts as was cp45, indicating that these three L protein mutations combine to specify a level of attenuation similar to that of cp45 and thus are the major contributors to the att phenotype.

Examination of the double mutants showed that two of them, r942/1558 and r942/992, were more attenuated than viruses bearing each individual mutation, consistent with the idea that the effects of the individual mutations are additive. However, similar to the situation described above for the ts phenotype, the level of attenuation of the r992/1558 virus in the upper respiratory tract was lower than that observed for viruses bearing each individual mutation. Thus, in two situations, illustrated by r992/1558 and r942/992/1558, the effect of times, producing three fragments (1,590, 922, and 439 bp; the latter species was not retained on the gel shown). Mutant DNA encoding the Leu-to-Phe mutation in position 922 (lanes 7 and 8) also have this site ablated and are therefore resistant to cleavage by EaeI. (B) Analysis with BsrI. BsrI cuts the wt RT-PCR product (lane 8) two lengths are indicated for several marker bands (in base pairs).
combining mutations is more complex than simple addition of their individual effects.

DISCUSSION

Reverse-genetics systems are providing powerful new tools for the characterization of attenuating mutations present in existing vaccines and for the development of new vaccine viruses (27). Recently, such systems have been developed for several members of the virus order Mononegavirales, and this new capability is changing the manner in which new vaccine viruses are being developed (5, 9, 14, 17, 23, 25, 29, 31). An excellent example of this new capability involves the characterization and generation of live attenuated vaccines for RSV (7). One approach involved the generation of a series of live attenuated RSV strains by the conventional techniques of passage in tissue culture or chemical mutagenesis, followed by nucleotide sequence analysis of promising vaccine candidates to identify putative attenuating mutations. The putative attenuating mutations were introduced singly and in combination into full-length antigenic cDNA encoding the RSV wt parent virus, followed by the characterization of recovered viruses to identify phenotypic changes associated with each mutation alone and in combination (7, 20). Another approach is to create de novo attenuating mutations which had not been found previously in nature. The successful application of this is exemplified by the identification of the attenuating effect of the deletion of the small hydrophobic gene of RSV (3). It is now possible to assemble a menu of attenuating mutations by using these two approaches and to combine the mutations from this menu into a live attenuated recombinant RSV virus via cDNA intermediates. The findings in the present study represent our initial results from applying these principles to the development of cDNA-based vaccines for the human parainfluenza viruses.

The recovery of a recombinant version of the JS strain of PIV3, the parent of cp45, has allowed us to begin to identify the mutations in cp45 that specify the ts and att phenotypes (9). The first step was to completely sequence the wt parent and cp45, the most promising of several cp derivatives (33, 34). Previous studies also suggested that cp45 contains ts and non-ts attenuating mutations and indicated that the att phenotype was stable after replication in vivo (15, 16, 22). The present study demonstrates that each of the three amino acid substitutions in the L protein of cp45 independently specifies both the ts and att phenotypes. However, none specifies the ca phenotype. Most importantly, a recombinant virus possessing all three L protein amino acid substitutions was as attenuated in the upper and lower respiratory tracts of hamsters as cp45 and was almost as temperature sensitive as cp45. These findings therefore identify the three L protein mutations as major contributors to the ts and the att phenotypes of cp45. The finding that these independent mutations in the L protein contribute to the ts and att phenotypes is a partial explanation for the observed stability of the ts and att phenotypes of this virus following replication in vivo (15). This is the first attenuating mutation or set of mutations that has been identified for PIV3 and thus begins our assembly of a menu of attenuating mutations for PIV3. The level of temperature sensitivity exhibited by each rPIV3 was consistent with its level of attenuation in vivo, showing that this in vitro marker is a useful predictor of the level of replication in vivo specified by these polymerase mutations.

It might have been predicted that combining the individual ts mutations would yield increased levels of temperature sensitivity and attenuation, as has been observed for influenza A virus (35). Indeed, this was observed in several instances in the present study. For example, the double mutants r942/992 and r942/1558 and the triple mutant r942/992/1558 were more temperature sensitive and attenuated than viruses bearing the individual mutations. Unexpectedly, the third double mutant, r992/1558, was less temperature sensitive and attenuated than either of the single mutants r992 or r1558. In this instance, the effect of combining the mutations was to reduce, rather than increase, the level of temperature sensitivity of the virus. Similarly, the triple mutant was less temperature sensitive and attenuated than r942/992 and marginally less attenuated than r942/1558. Thus, the level of temperature sensitivity and attenuation achieved by the stepwise combination of mutations was cumulative except in two cases, r992/1558 and r942/992/1558, where the effect of combining mutations was more complex than the effect of the sum of the individual mutations. This is suggestive of an interaction between the mutations.

The mechanisms by which these three mutations contribute to the ts and att phenotypes remain unknown. A ts mutation typically is thought to act through destabilization of protein stabilization and repression of viral[

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus titer (log_{10} TCID_{50}/g) [mean ± SE] in:</th>
<th>nasal turbinates</th>
<th>lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt 1</td>
<td>Expt 2</td>
<td>Expt 1</td>
</tr>
<tr>
<td>rPIV3 wt</td>
<td>7.4 ± 0.16</td>
<td>6.4 ± 0.11</td>
<td>5.1 ± 0.49</td>
</tr>
<tr>
<td>r942</td>
<td>6.6 ± 0.17</td>
<td>5.6 ± 0.19</td>
<td>3.0 ± 0.78</td>
</tr>
<tr>
<td>r992</td>
<td>4.4 ± 0.16</td>
<td>3.1 ± 0.11</td>
<td>3.1 ± 0.11</td>
</tr>
<tr>
<td>r1558</td>
<td>3.8 ± 0.40</td>
<td>4.3 ± 0.34</td>
<td>4.3 ± 0.34</td>
</tr>
<tr>
<td>r942/992</td>
<td>&lt;1.5 ± 0.10</td>
<td>&lt;1.5 ± 0.08</td>
<td>&lt;1.5 ± 0.10</td>
</tr>
<tr>
<td>r942/1558</td>
<td>2.9 ± 0.23</td>
<td>1.8 ± 0.17</td>
<td>1.8 ± 0.17</td>
</tr>
<tr>
<td>r992/1558</td>
<td>5.7 ± 0.16</td>
<td>4.8 ± 0.18</td>
<td>3.2 ± 0.57</td>
</tr>
<tr>
<td>r942/992/1558</td>
<td>3.9 ± 0.15</td>
<td>4.6 ± 0.24</td>
<td>&lt;1.5 ± 0.10</td>
</tr>
<tr>
<td>cp45</td>
<td>4.1 ± 0.27</td>
<td>5.1 ± 0.17</td>
<td>1.6 ± 0.08</td>
</tr>
</tbody>
</table>

* Groups of six hamsters each were intranasally administered 10^5.4 PFU of virus in a 0.1-ml inoculum, and the lungs and nasal turbinates were harvested 4 days later.

The cp45 virus is a biologically derived virus; the other viruses are recombinants.

TCID_{50}, 50% tissue culture infectious dose.

TABLE 3. Levels of replication in the upper and lower respiratory tracts of hamsters bearing one, two, or three cp45 L protein amino acid substitutions and of wt rPIV3 (JS strain) and cp45**

* Values are means of experiments unless indicated otherwise. Underlined numbers represent the shutoff temperature of plaque formation, which is defined as the lowest restrictive temperature at which a 100-fold reduction in titer is observed, compared to the titer at 32°C.
* Plaque were of pinpoint size.
* Value is from a single experiment.
* The cp45 virus is a biologically derived virus, and each of the other viruses tested is a recombinant.

** cp45 is a biologically derived virus; the other viruses are recombinants.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus titer (log_{10} TCID_{50}/g) [mean ± SE] in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>-------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Expt 1</td>
</tr>
<tr>
<td>rPIV3 wt</td>
<td>7.4 ± 0.16</td>
</tr>
<tr>
<td>r942</td>
<td>6.6 ± 0.17</td>
</tr>
<tr>
<td>r992</td>
<td>4.4 ± 0.16</td>
</tr>
<tr>
<td>r1558</td>
<td>3.8 ± 0.40</td>
</tr>
<tr>
<td>r942/992</td>
<td>&lt;1.5 ± 0.10</td>
</tr>
<tr>
<td>r942/1558</td>
<td>2.9 ± 0.23</td>
</tr>
<tr>
<td>r992/1558</td>
<td>5.7 ± 0.16</td>
</tr>
<tr>
<td>r942/992/1558</td>
<td>3.9 ± 0.15</td>
</tr>
<tr>
<td>cp45</td>
<td>4.1 ± 0.27</td>
</tr>
</tbody>
</table>
folding, an effect which is aggravated by increased temperature. The finding that the level of temperature sensitivity was a good predictor of attenuation implies that perturbation of protein folding is involved in both phenotypes. This also provides a reasonable basis for interpreting the interaction between mutations. For example, the finding that each of the mutations at positions 992 and 1558 alone resulted in greater temperature sensitivity and attenuation than they did together could mean that each mutation partially suppresses the destabilizing effect of the other. Interestingly, the tyrosine-to-histidine mutation at position 942, arguably the most conservative substitution at position 1558, affected multiple polymerase activities (18).

The L polymerase of PIV3 is a large polypeptide, 2,233 aa in length, and is thought to be a multifunctional protein that consists of multiple domains, including those required for association with the P protein, RNA binding, RNA polyadenylation, RNA transcription, and RNA replication (4). The amino acid substitutions in L at positions 942 and 992 are located near regions that are well conserved among other members of the Paramyxoviridae family (2, 13). The mutation at position 1558 is in a region of the polymerase that appears to have less sequence identity with other L polymerases. Although the mechanism by which the ts phenotype is conferred by the triple amino acid substitution in L is not known, it may be that multiple activities are affected or that the interplay between the various activities may be affected, as has been suggested for a vesicular stomatitis virus L gene mutant (19). In fact, substitution mutations in the Sendai virus L polymerase at aa 1571, a region analogous to that of the cp5 Thr-to-Ile substitution at position 1558, affected multiple polymerase activities (18).

The L gene containing mutations yielding all three attenuating amino acid substitutions can now be used alone or in conjunction with other attenuating mutations as they are identified or created in a cDNA-derived vaccine for PIV3. Furthermore, the attenuating L gene can now be transferred to the recently isolated PIV1-PIV3 chimeric recombinant virus bearing the protective antigens, the HN and fusion glycoproteins, of PIV1 on a background of PIV3 genes (35a). In this way, it should be possible to rapidly generate a live attenuated PIV1 candidate vaccine.

ACKNOWLEDGMENTS

We thank R. Chanock for critical review of the manuscript and F. Wood, J. Siew, and F. Davoodi for technical assistance.

REFERENCES


PHENOTYPES OF PIV3 cp5 Vaccine Candidate


ACKNOWLEDGMENTS

We thank R. Chanock for critical review of the manuscript and F. Wood, J. Siew, and F. Davoodi for technical assistance.
purified F glycoprotein and challenged with RSV 3-6 months after immunization. Vaccine 8:497–502.


