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Characterization of a Live, Attenuated Human Parainfluenza Type 3 Virus Candidate Vaccine Strain

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Characterization of a temperature-sensitive and live, attenuated human parainfluenza type 3 virus strain (cp45) grown at a permissive temperature (32°C) suggested that the virus efficiently multiplies in cell lines and retains antigenic and functional properties of the envelope glycoproteins. When grown at a nonpermissive temperature (39.5°C), the cp45 virus exhibited poor replication; however, shifting to a permissive temperature allowed virus growth. Although at a nonpermissive temperature virus polypeptide synthesis was significantly reduced, the hemagglutinin-neuraminidase and fusion glycoproteins were transported to cell surfaces and retained their characteristic biologic activities. Studies on mRNA synthesis from the P protein gene suggested a poor transcriptional activity of the cp45 virus at a nonpermissive temperature. Results from this study indicate that the temperature sensitivity of cp45 virus is related to altered transcriptional activity and a marked reduction in virus polypeptide synthesis.

Human parainfluenza viruses (HPIVs), members of the *Paramyxoviridae* family, are responsible for serious lower respiratory tract infections in infants (4, 37). HPIV type 3 (HPIV-3) often infects young infants less than 6 months of age and causes bronchiolitis, pneumonia, or croup. It is second only to respiratory syncytial virus as a cause of lower respiratory tract infection in neonates and young infants. The HPIV-3 genome contains a single-stranded negative-sense RNA of approximately 15,000 nucleotides (27, 33) and encodes at least six structural proteins (3′-nucleocapsid protein [NP]-P[+C]-matrix protein [M]-fusion protein [F]-hemagglutinin-neuraminidase protein [HN]-L-5′) (30, 31). The genome RNA and its full-length complement, the antigenome RNA, are found both in the virion and in the infected cell as an RNase-resistant nucleocapsid because of the tight association of the major NP with the RNA (12). Two other viral proteins, P and L, are associated less tightly with the nucleocapsid and function as subunits of the RNA-dependent RNA polymerase. Three additional viral proteins, HN, F, and M, are associated with the lipid envelope of the virion. Both HN and F glycoproteins are responsible for initiation and progress of the infection process (11, 15) and have been shown to induce neutralizing antibodies and correlate with protection from infection (16, 24). On the other hand, HN, F, NP, and M proteins have been shown to induce cytotoxic T cells (1, 20). HPIV-3-mediated immunoregulation of human T lymphocytes has been suggested to play an important role in the failure of the virus to induce lifelong immunity (29).

Cold-adapted and temperature-sensitive (*ts*) HPIV-3 mutants were generated by serial passages at a suboptimal temperature (2). Cold-adapted viruses were also temperature sensitive and attenuated for growth in the lungs and nasal turbinates of experimental animals. The reduction of virus replication was directly related to the cold passage level of the virus (3, 6, 9). cp45 virus was also evaluated in seronegative chimpanzees and found to be highly attenuated in both the upper and lower respiratory tracts and showed resistance to challenge infection (13). cp45 virus is currently under evaluation in humans as a candidate vaccine against HPIV-3 infection. In this study, we determined the basis of temperature sensitivity of the cp45 candidate vaccine strain. Our results suggest that the transcriptional activity of the vaccine strain is temperature sensitive and significantly reduces the overall protein synthesis and replication of the virus at the nonpermissive temperature.

Temperature-dependent replication of cp45. cp45 virus was adsorbed at 32°C for 1 h and grown at 39.5°C for 24 h. Infected cells were then shifted to 32°C for growth for an additional 24 h. The virus titer in the culture supernatant was determined by plaque assay in L-132 cells by a method similar to that described previously (2). Infected cell monolayers were either stained with hematoxylin-eosin Y or overlaid with 0.9% agar and 0.005% neutral red (21) for visualization of viral plaques. The results from at least three independent experiments showed consistent virus recovery (less than fivefold variation). Comparison of cp45 and wild-type (JS) virus replication in L-132 cells at permissive (32°C) and nonpermissive (39.5°C) temperatures in a temperature-shift assay is shown in Table 1. Normal growth of both the viruses was observed at the permissive temperature. However, poor replication (reduction of $\sim 10^6$-fold) of cp45 was observed when the virus was adsorbed on L-132 cells at 39.5°C and incubated at the same temperature. However, the virus showed some replication upon changing the incubation temperature from 39.5 to 32°C after 24 h. On the other hand, the wild-type virus exhibited similar replication at 39.5 and 32°C. cp45 virus replicated upon temperature shift from 39.5 to 32°C demonstrated the characteristic temperature-sensitive phenotype. This indicated an absence of the revertant virus at 39.5 or 32°C. Results from the temperature-shift experiment suggest that, unlike the parent wild-type virus, the cp45 virus temporarily lost its normal replication property upon incubation at the nonpermissive temperature.

Epitope recognition by monoclonal antibodies. Antigenic relatedness of cp45 and the wild-type parent virus strain was initially compared by hemagglutination (HA) inhibition and neutralization assays using a monospecific rabbit antiserum to affinity-purified HPIV-3 HN glycoprotein (23). Rabbit an-

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TABLE 1. Comparison of yields of cp45 and parent wild-type (WT) viruses in a temperature-shift experiment

Virus strain	Incubation temp (°C)	Virus yield[a]
cp45	32	2.9×10^7
WT	32	1×10^7
cp45	39.5	1.2×10
WT	39.5	8×10^6
cp45	39.5→32[b]	2×10^3
WT	39.5→32[b]	1.5×10^7

[a] Virus yields in L-132 cells, infected at similar multiplicities of infection, are expressed after 48 h of incubation.
[b] Temperature shift.

TABLE 2. Comparison of neuraminidase activities of cp45 and parent wild-type (WT) viruses

Virus strain	Incubation temp (°C)	HA unit used	Neuraminidase activity with[a]:	
			Fetuin	Neuramin-lactose
WT	39.5	512	0.24	3.60
cp45	39.5	512	0.03	0.83
WT	32	2,048	1.11	2.30
cp45	32	2,048	0.67	2.30

[a] Results are presented as optical density values at 549 nm.

ti-HN showed similar HA inhibition activities and neutralization titers (within twofold variation) with both the virus strains. Subsequently, representative anti-HN and anti-F monoclonal antibodies recognizing distinct antigenic sites of the HN and F glycoprotein molecules were tested by enzyme-linked immunosorbent assay (ELISA) following a method similar to the one described previously (21). Dynatech polyvinyl plates (Immulon I) were coated with 1 μg of freeze-thaw disrupted virions per well. Monoclonal antibodies were tested at twofold serial dilutions for each virus strain (cp45 and the wild type), and the results were compared with the linear slopes of the reactivity pattern. The results are presented (Fig. 1) as the mean optical densities from three independent experiments, showing variations within 0.05 to 0.08. The antibodies recognized the HN and F glycoproteins of cp45 virus and by ELISA showed a titers similar to that of the wild-type virus. This suggests that the antigenic sites of cp45 virus were not altered as a result of its adaptation for growth at 20°C.

Biological activities of cp45 virus. We examined whether the transport of cp45 virus HN and F glycoproteins to the cell surface is blocked under nonpermissive conditions. Infected L-132 cells were tested 24 h after infection by immunofluorescence with specific monoclonal antibodies to HN and F. Confluent monolayers of L-132 cells were grown on coverslips, infected with the virus, and incubated at 32 or 39.5°C. At 24 h postinfection, cells were washed with phosphate-buffered saline and tested with monoclonal antibodies following a procedure similar to the one described earlier (21). At both incubation temperatures (32 and 39.5°C), cp45-infected cells showed immunofluorescence on the cell surface (data not shown). The result from this study indicates that the cp45 virus glycoproteins are processed and transported to the cell surface even at the nonpermissive temperature.

cp45 virus was grown in L-132 cells at 32 or 39.5°C, and the virus-infected cell homogenate was analyzed for neuraminidase activities. Briefly, 100 μl of 0.2 M sodium acetate buffer (pH 5.5) was mixed with an equal volume of infected cell homogenate with a known number of HA units. Then, 0.1 ml of bovine fetuin (15 mg/ml, type IV; Sigma Chemical Company, St. Louis, Mo.) dissolved in the same buffer was added to the reaction mixture, and the mixture was incubated at 37°C overnight. The amount of released neuraminic acid in the reaction mixture was determined as described earlier (21). Wild-type parent virus was also included in this study for comparison. The neuraminidase property of the cp45 virus incubated at the nonpermissive temperature when tested with two different molecular-size substrates showed at least fourfold lower activity than cells infected with the wild-type virus (Table 2). The results from this study provided information on the reduced neuraminidase activity of cp45 virus at the nonpermissive temperature, which may affect the release of the progeny virus particles from the infected cell surface.

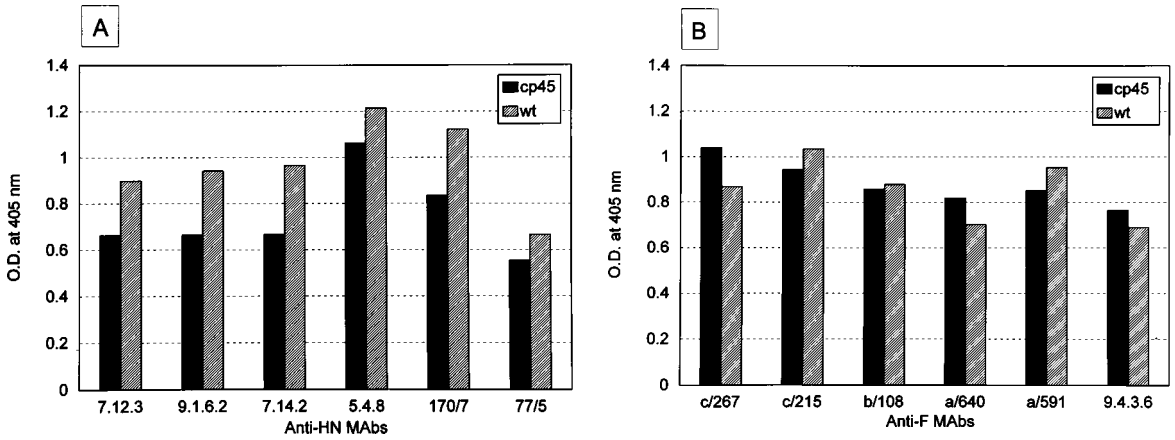

FIG. 1. Reactivities of cp45 and parent wild-type (wt) viruses with monoclonal antibodies (MAbs) to HN (A) and F (B) glycoproteins by ELISA. Results are presented as the mean optical densities (O.D.) from three different experiments at a particular dilution of the antibodies (1:800) for comparison. Characteristics of the monoclonal antibodies 7.12.3, 9.1.6.2, 7.14.2, 5.4.8, and 9.4.3.6 defining antigenic sites of HPIV-3 (strain 47885) have been previously described (22, 23). Monoclonal antibodies 170/7, 77/5, c/267, c/215, b/108, a/640, and a/591 were kindly provided by Judy Beeler (World Health Organization Reagent Bank). The reactivities of these monoclonal antibodies have been previously described (7, 8).

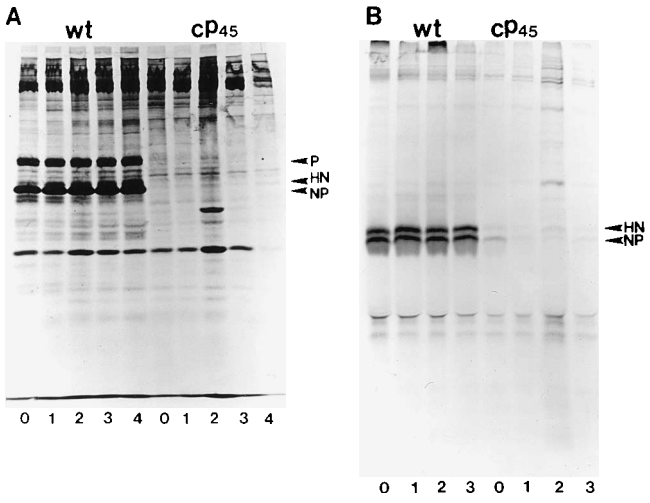

FIG. 2. Kinetics of viral protein synthesis by pulse-chase experiment. Cells infected with the wild-type or cp45 virus were grown at 39.5°C for 24 h and pulsed with ^{35}S-protein label for 1 h. Labeled cell lysates were immunoprecipitated with rabbit anti-HPIV-3 (A) or pooled monoclonal antibodies to HN and NP of HPIV-3 (B) at the indicated time points under each lane (0, 1, 2, 3, and 4 h). The positions of the virus polypeptides, indicated on the right, were determined on the basis of the patterns reactivity with the antibodies and molecular size based on the migration of molecular weight markers (data not shown).

cp45 virus grown at 32°C was pelleted by ultracentrifugation and used for an HA assay to test the functional property of the HN glycoprotein following a procedure similar to that described earlier (21). As the cp45 virus showed extremely poor growth at 39.5°C, we tested the HA activity of infected cell homogenates following incubation at the nonpermissive temperature. Results show detectable HA activity of the cp45 virus grown at the permissive or nonpermissive temperature. cp45 virus-infected LLC-MK_2, Vero, and L-132 cells also showed formation of multinucleated giant cells or syncytium formation, a characteristic of virus fusion activity (23), when grown at 32°C. However, fusion activity was significantly reduced upon incubation of cp45 virus-infected cells at 39.5°C, probably because of poor replication of the virus at the nonpermissive temperature.

Polypeptide synthesis. cp45 virus polypeptide synthesis was analyzed by a pulse-chase experiment followed by immunoprecipitation with a hyperimmune rabbit antiserum to HPIV-3 or monoclonal antibodies to HN and NP (21). Briefly, virus-infected cells were grown at 39.5 or 32°C for 24 h and pulsed with ^{35}S-protein label (Amersham Corporation, Arlington Heights, Ill.) for 1 h. Labeled cell lysates were immunoprecipitated after a chase of 0, 1, 2, 3, and 4 h with hyperimmune rabbit antiserum to HPIV-3 or with a pool of anti-HN and anti-NP monoclonal antibodies (21). Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by autoradiography. The results show a major difference in the synthesis of viral proteins between the wild-type and cp45 virus. Synthesis of wild-type virus polypeptides during the pulse period was not processed or modified further during the 4-h chase period (Fig. 2). On the other hand, cp45 virus polypeptide synthesis appeared to be extremely weak or almost undetectable. However, synthesis of cp45 and wild-type virus polypeptides was found to be similar when cells were grown at 32°C (data not shown).

Virus-specific mRNA synthesis. During reproduction of paramyxoviruses in the cytoplasm of infected cells, the nucleocapsid (RNA-NP) serves as a template for transcription by the viral RNA polymerase to synthesize individual mRNA to encode viral polypeptides. In order to determine the overall viral transcriptional activity at the nonpermissive temperature, mRNA synthesis from the P protein gene of cp45 virus was studied by reverse transcription-PCR for comparison with that of the wild-type virus, by a method similar to that described by Dallman and Porter (10). Briefly, L-132 cells were infected with cp45 or wild-type virus at a similar multiplicity of infection. After virus adsorption at 32°C, cells were grown at 39.5°C for 24 h. Infected cells were treated with actinomycin D (10 μg/ml) for 14 h, and RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction (5). Amplification of the P mRNA was done by reverse transcription-PCR from the same quantities of total RNA (10 μg) and virus-specific (31) sense (CCAACAACAACTCCCAGATC, nucleotide positions 2740 to 2759) and anti-sense (TGCCTCCATAAGTG GGTCAA, nucleotide positions 3280 to 3299) synthetic oligonucleotide primers, by using reagent concentrations similar to those previously described (25). The amplification reaction was performed by using an automatic thermocycler (Perkin-Elmer Cetus), with cycling parameters of denaturation at 94°C for 1 min, primer annealing at 50°C for 1.5 min, and primer extension at 72°C for 2 min. As an internal standard, amplification of the actin mRNA was similarly done by reverse transcription-PCR by using the same RNA preparations and β-actin gene-specific sense (GCATGGAGTCCTGTGGCATCCACG, nucleotide positions 2563 to 2586) and anti-sense (CTAGA AGCATTTGCGGTGGACGAT, nucleotide positions 2977 to 3000) primers. A plasmid DNA containing the P protein gene of HPIV-3, kindly provided by Mark S. Galinski (The Cleveland Clinic Foundation, Cleveland, Ohio), was also used as a positive control in the PCR amplification. A PCR-amplified ~559 bp fragment from the P protein gene plasmid DNA was isolated by electrophoresis in a 0.8% agarose gel. The DNA band was excised, eluted by using an ultrafree MC column (Millipore Corporation, Bedford, Mass.), and radiolabeled with [α-^{32}P]dCTP by the random primed oligonucleotide labeling method by using a commercially available kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), for use as a probe. An actin probe was similarly prepared for use in Southern hybridization.

mRNA synthesis was tested by comparing the levels of message between the two RNA preparations in a parallel experiment. The reaction products from different cycles of PCR amplification were analyzed by agarose gel electrophoresis followed by ethidium bromide staining and showed a significant difference in the levels of message generated from cp45 and wild-type virus. On the other hand, similar message levels of the actin gene, studied as an internal control, was observed for the RNA isolated from cp45 and wild-type virus-infected cells (data not shown). A similar observation was noted following Southern hybridization of the electrophoresed DNA. The typical amplification profile of the PCR products from viral mRNA is shown in Fig. 3A. A semiquantitative approach was taken for the estimation of the differences between these messages by slot blot hybridization with fourfold dilutions of a single cDNA sample. A representative example of the results, shown in Fig. 3B, further indicates differences in the message of the P gene from cp45 and wild-type virus-infected cells at 15 and 20 cycles of PCR amplification. Message from the P protein gene of cp45 virus was estimated to be approximately 17% of that of wild-type virus by PhosphorImaging analysis. Results from this study suggest that the cp45 virus produces low levels of mRNA at a nonpermissive temperature.

In this report, we have demonstrated that the temperature-sensitive property of the cp45 candidate vaccine strain is asso-

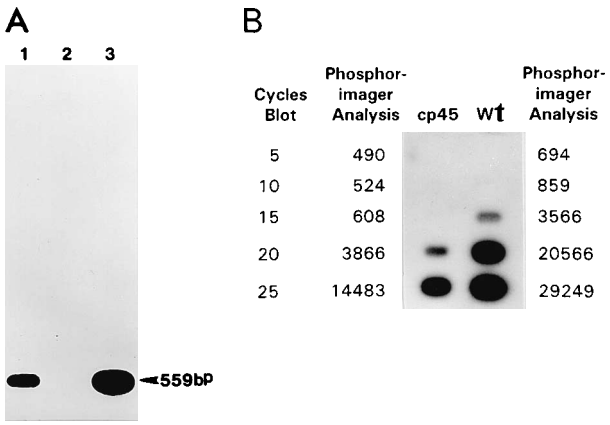

FIG. 3. PCR amplification and comparison of mRNA levels of P protein gene between cp45 and parent wild-type viruses. PCR-amplified products of the cDNA were analyzed by Southern hybridization or slot blot analysis. (A) DNAs after 15 cycles of amplification from wild type (lane 1), cp45 (lane 2), and plasmid DNA containing the P protein gene (lane 3) were subjected to 0.8% agarose gel electrophoresis and Southern blot hybridization with a P protein gene probe. The size of the amplified DNA, indicated by an arrow on the right, was calculated on the basis of migration of ϕX174/*Hae*III-digested DNA marker (data not shown). (B) Results of slot blot hybridization and PhosphorImaging analysis using the Molecular Dynamics PhosphorImager.

ciated with poor transcriptional activity of the virus at a nonpermissive temperature. Virus mRNA synthesis is markedly reduced at a nonpermissive temperature, and as a result the polypeptide synthesis and virus growth are significantly affected. Our results suggest that although the transcriptional activity of cp45 is reduced, virus glycoproteins are transported to infected cell surfaces and have limited viral morphogenesis, indicating poor virus growth at the nonpermissive temperature. The vaccine strain would not be useful if the virus did not undergo limited replication in vaccinated humans, as this is necessary to stimulate the appropriate immune responses. Antigenic sites of the envelope glycoproteins, previously defined by panels of monoclonal antibodies (7, 8, 22, 23), were reactive similar to the parent wild-type virus, and the biological activity of the virus envelope glycoproteins appeared to remain unaffected as a result of cold adaptation. However, the biological activities of the individual functional domains of the virus proteins were not investigated in this study.

Characterization of *ts* Sendai virus mutants have suggested a defect in the HA protein affecting infectivity of progeny virus particles when grown at a nonpermissive temperature (17, 34). *ts* mutants of Newcastle disease virus have been shown to be sensitive for fusion and hemadsorption properties (18, 19, 35). At the permissive temperature, the biological properties of *ts* RNA^+ mutants showed lower HA, neuraminidase, and hemolysis activities compared with those of the wild-type virus. However, these mutants showed infectivities similar to that of the wild-type virus. A recent study (28) of a *ts* mutant of influenza B virus suggested that viral proteins were produced at the nonpermissive temperature. However, the *ts* mutant did not show enzymatic or HA activity but produced noninfectious virus particles which become infectious after treatment with trypsin. The results from our study with cp45 do not suggest detectable changes in the biological properties of the virus glycoproteins when grown at the permissive or nonpermissive temperature. Comparison of the nucleotide and predicted amino acid sequences of cp45 and the parent wild-type virus suggested seven amino acid substitutions in genes encoding four different polypeptides, M, F, HN, and L (32). Apart from other changes, three unique substitutions in the L protein of cp45 have been noted. Furthermore, the 3′ leader region, important in gene regulation and in virus propagation, showed nucleotide changes.

Characterization of a cold-adapted influenza A virus vaccine strain suggested conformational changes in the RNA structure facilitating advantageous growth at 25°C (14). Mutations in the noncoding regulatory regions, as a result of the attenuation process, have been suggested to affect transcription and/or replication of polio viruses (26, 36). It appears that the changes in the 3′ leader region and amino acid substitutions in the L protein of cp45 may play a role in the down-regulation of the transcriptional activity at the nonpermissive temperature. This may be due to a conformational change of the polymerase or some unknown factor(s). The molecular mechanism of temperature sensitivity in cp45 virus by complementation assay using L and/or P protein genes from the wild-type virus and identification of the attenuating lesions will facilitate further characterization of this promising live virus vaccine strain.

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