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Two Distinct Human Parainfluenza Virus Type 1 Genotypes Detected during the 1991 Milwaukee Epidemic

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The extent of genetic and antigenic variation found in a population of human parainfluenza virus type 1 (HPIV-1) during a single local epidemic was investigated. Fifteen HPIV-1 strains isolated from children in 1991 were analyzed. Nucleotide sequence variation in the hemagglutinin-neuraminidase protein (HN) gene demonstrated two distinct genotypes (genotypes C and D). Unique patterns were identified involving 62 nucleotide and 10 amino acid positions. These patterns represented 40% of all mutations within the HN gene. The remaining mutations were randomly distributed, and 74% involved only one (55%) or two isolates. Genotypes were statistically different from each other at both the nucleotide ($P = 0.001$) and amino acid ($P = 0.001$) levels and demonstrated unique potential N-linked glycosylation patterns. Thirty-eight monoclonal antibodies (MAbs) made to four different viral proteins (22 HN, 2 fusion [F], 1 phosphoprotein, and 13 nucleoprotein) (originating from two different genotypes [genotypes A and D]) were compared for their ability to bind to the clinical isolates in enzyme-linked immunosorbent assays (ELISAs) and hemagglutinin-inhibition (HI) assays. Twenty-one MAbs bound well to all clinical isolates in ELISAs and HI assays. The remaining 17 MAbs showed variation in all four structural proteins. Three HN MAbs demonstrated genotype C- and D-specific antigenic and neutralization differences. Evolutionary analysis using parsimony methods confirmed the differences between the two genotypes. No differences in either clinical presentation or disease severity between the two genotypes were found. Geographically localized HPIV-1 epidemics can be caused by at least two distinct genotypes with minor but specific antigenic changes. The clinical and immunologic roles of HPIV-1 genotypes have not been determined.

Human parainfluenza virus type 1 (HPIV-1) is a major cause of lower respiratory tract infections in infants, young children, and the immunocompromised (8, 10). This virus has worldwide distribution and probably contributes significantly to childhood mortality in the developing world (8, 10). In the United States, we have demonstrated significant morbidity and cost attributable to HPIV-1 epidemics (12). During these epidemics, approximately 100,000 children less than 5 years old are seen in emergency rooms and approximately 35,000 are hospitalized at a combined cost of approximately $90,000,000 (12). Currently, there is no specific therapy or vaccine for HPIV-1.

We recently reported that HPIV-1 collected over a 26-year period in a single city demonstrated different genotypes and that one of these genotypes (genotype A) had genotype-specific antigenic markers that could be detected by using monoclonal antibodies (MAbs) and human sera (7, 14). Subsequently, others have found similar antigenic changes in HPIV-1, and one report failed to find genotypes or antigenic markers over a 9-year period (15, 17). The significance of these findings is still to be determined. If children make antibodies directed at one HPIV genotype and not another, then it is possible that immunization with one strain of HPIV-1 might not protect against others. Furthermore, HPIV-1 genotypes may be important in the epidemiology and pathophysiology of HPIV-1. Vaccine strategies to prevent HPIV-1 infection may need to take this information into account. Using 38 MAbs and sequence analysis of the hemagglutinin-neuraminidase (HN) gene, we determined antigenic and genetic variation in 15 isolates of HPIV-1 collected in the fall of 1991.

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MATERIALS AND METHODS

Cells and virus. Monkey kidney (LLC-MK_2) cells were grown in Eagle's minimal essential medium supplemented with 5% fetal bovine serum. Confluent monolayers were infected with HPIV-1 at a multiplicity of infection of 0.1. Serum-free HB101 medium (Irvine Scientific, Santa Ana, Calif.) with 2.5 μg of acetylated trypsin per ml was added. The virus was harvested, concentrated, assayed, and stored as previously described (13). HPIV-1 (DC-1957, HA-2, strain C39; American Type Culture Collection, Rockville, Md.) was used as the type strain. The other 15 clinical isolates were recovered during the 1991 HPIV-1 epidemic in Milwaukee, Wis. The viruses were recovered from children less than 6 years old between September and December (12).

MAbs. Mice (CAF1) were infected by intravenous injection of detergent-disrupted HPIV-1 from the 1991 epidemic (CI-58). Hybridomas of mouse spleen cells and SP2/0-Ag14 myeloma cells were produced, and their supernatants were tested (5). MAbs specific for HPIV-1 structural proteins were identified by radioimmunoprecipitation assays (RIPAs). More than 80 HPIV-1 (1991)-specific MAbs were made (91 MAbs). MAbs directed against the 1957 type strain (57 MAbs) have been previously reported (7). Samples of ascitic fluid containing MAb were aliquoted and frozen at −70°C until use.

RIPAs. RIPAs were performed as previously described (11).

ELISAs. ELISAs were performed as previously described (7, 11). Each well of the ELISA plates was coated with 1.0 μg of purified virus as determined by a protein dye assay (Pierce, Rockford, Ill.). Serial twofold dilutions of each ascitic fluid or serum sample were tested in duplicate and the titer was defined as the antibody dilution that yielded an A_{405} of >0.1 on a spectrophotometer (EL 311; Bio-tek, Winooski, Vt.). A titer change of more than 4 (i.e., 16-fold difference) was arbitrarily chosen as being significant.

HI assay. Hemagglutinin inhibition (HI) assays were performed by the standard methods (7, 28), except that the guinea pig erythrocyte solution had a starting hemagglutinin titer equal to 4. The titer change criterion described above was used to assign statistical significance. HI titers of less than 64 were considered nonspecific activity.

Microneutralization assay. MAbs were twofold serially diluted in HB101 (serum-free medium). Portions (75 μl) of each dilution were pipetted into three wells of a 96-well sterile flat-bottom tissue culture plate. HPIV-1 (60 PFU per well in 25 μl of HB101 was added to each MAb dilution to be tested. Twenty-five microliters of HB101 was added to each control well. After 2 h at room temperature, 100 μl of LLC-MK_2 cells (200,000 cells per ml) with 2 to 5 μg of acetylated trypsin per ml in the final volume was added to each test well. Plates were loosely wrapped in plastic film and incubated at 34°C in a CO_2 incubator for 3 to 5 days. The fluid from each well was then transferred by a multiwell pipette into a 96-well ELISA plate, and a standard ELISA was performed as previously

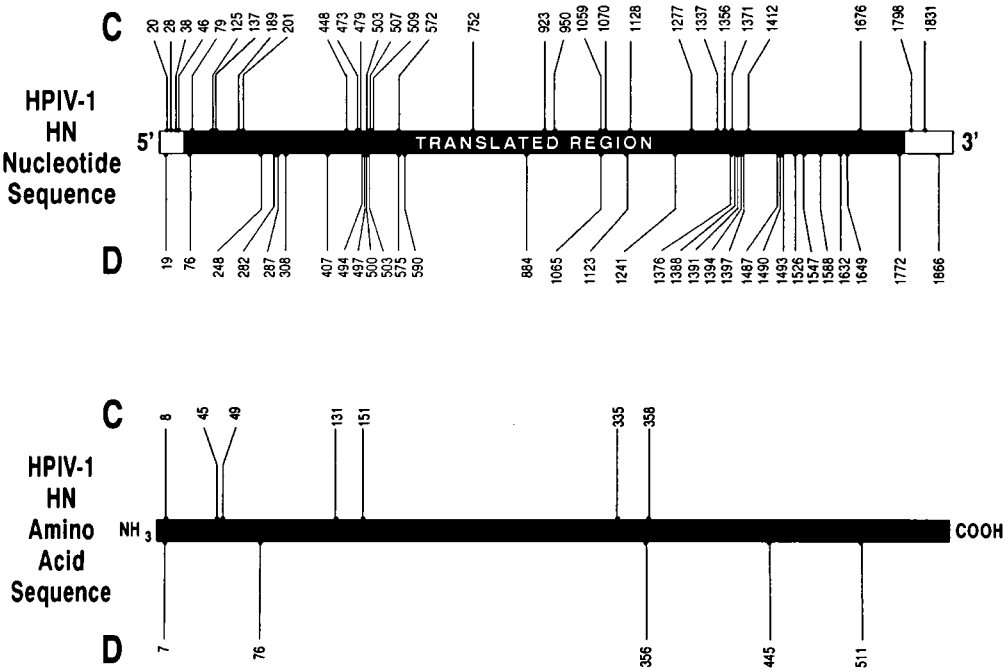

FIG. 1. Locations of genotype-specific mutations on the HN nucleotide and amino acid sequences of HPIV-1.

described for antigen detection. Controls included virus only, MAb only, and no virus or MAb (cells only).

Nucleic acid sequence analysis. Genomic RNA was extracted, purified, and sequenced as previously described (14). Sequencing data were analyzed with Genetics Computer Group software from the University of Wisconsin, Madison (6). Evolutionary relationships were calculated by parsimony methods available in Genetics Computer Group and Phylip 3.5 (University of Washington) software.

Statistical analysis. The combined homogeneity of paired nucleic acid or amino acid differences within each subgroup was compared with the homogeneity of paired differences between subgroups by a permutation technique to give a measure of central tendency.

Nucleotide sequence accession numbers. Nucleotide sequences have been reported to GenBank (accession numbers pending).

RESULTS

The extent of genetic variation that exists within an epidemic of HPIV-1 was investigated by sequencing the HN gene from 13 clinical isolates. The nucleotide sequences were then aligned and compared. The HN genes of the 13 clinical isolates were each 1,894 bp long with an open reading frame of 1,725 bp without additions or deletions. The 5′ and 3′ untranslated regions were 56 and 113 bp long, respectively. Six base pairs could not be resolved after multiple attempts. Four of these base pairs were in noncoding regions of the genome, with 3 bp in one compressed area (bp 1845 to 1848).

Sequence variation identifies two distinct genotypes. There were 160 nucleotide positions (8.4%) where substitutions took place. Nineteen of these positions were in noncoding regions, leaving 8.2% (141 positions) on the coding portion of the HN gene. Unique nucleotide substitution patterns were found in 62 of 160 positions (approximately 40%). The rest appeared random. Seven clinical isolates had 30 mutations in common (genotype C), while six isolates (genotype D) shared 32 mutations (Fig. 1). In only 11 of 160 positions did strains in both genotypes have nucleotide substitutions in common. In 73 of the 98 non-genotype-specific positions, mutations took place in only one (55%) or two (19%) HPIV-1 isolates at a time.

The pairwise comparison of HPIV-1 nucleotide sequences demonstrates a significant difference between genotype C and D isolates. Genotype C isolates varied by 16 to 37 nucleotide substitutions (an average of 24), and genotype D isolates varied by 7 to 35 substitutions (an average of 24). However, genotype C isolates differed from D isolates by 78 to 100 (an average of 87 [$P = 0.001$]) nucleotide substitutions.

Predicted amino acid sequences. The predicted amino acid sequences for the HN proteins of the clinical isolates were aligned and compared. There were 40 mutations in 38 substituted positions of 575 amino acids on the HN protein. Genotype C isolates had seven unique mutations, and two of these mutations were in 6 of 7 isolates (Fig. 1). Genotype D had five unique mutations (Fig. 1). One half of the amino acid substitutions were conservative changes to amino acids with similar charges or polarities, but the other half consisted of changes to amino acids with different charges or changes from polar to nonpolar side groups. Furthermore, genotype C isolates had a change at amino acid 358 from an alanine to a proline which could significantly affect structure. In general, the HN structure appears to be highly conserved with all of the rest of the 28 proline and 18 cysteine amino acids conserved among the Milwaukee isolates.

The pairwise comparison of the amino acid sequences demonstrated patterns similar to those seen with the nucleotide sequences. Isolates within the same genotype had few amino acid differences (average for genotype C isolates, 1%; average for genotype D isolates, 1.4%); isolates from different genotypes had significantly more amino acid differences (average, 3.5% [$P = 0.001$]).

Distribution of nucleotide and amino acid substitutions. The distribution of genotype-specific nucleotide and amino acid substitutions along the HN gene and protein can be seen in Fig. 1. The first 18 and the last 28 nucleotides were conserved in all Milwaukee isolates and may have a regulatory function. There were several areas of increased mutation rate along the HN gene that differed between genotypes (e.g., bp 20 to 137 for genotype C and bp 248 to 308 and 1487 to 1649 for

genotype D). However, there were several areas on the HN gene where each genotype demonstrated similar yet unique mutations. Both genotypes had six mutations between bp 473 to 590 and three to five substitutions between bp 1337 to 1397.

Amino acid sequences demonstrate similar patterns. Each genotype had an early mutation (amino acids [aa] 8 and 7) and mutations at aa 356 or 358, but they differed in the locations of the rest of the amino acid substitutions. Genotype C isolates had four mutations in the first 151 aa, while genotype D isolates had only one mutation; genotype D isolates had two mutations in the last 130 aa, while genotype C isolates had none. The common areas of mutation may indicate areas that allow structural change and escape from immune detection without functional change.

Genotypes have different N-linked glycosylation sites. Nine potential N-linked glycosylation sites (Asn-X-Ser/Thr-X, where X means any amino acid except proline) were found between the 13 clinical isolates (4). The potential sites begin at aa 8, 19, 77, 173, 277, 361, 499, 511, and 551. All nine sites are located on the 1957 type strain. Eleven of the Milwaukee isolates had eight of these sites, and two strains had seven. These sites could be important in posttranslational modification of the HN protein. Genotype C isolates are all missing the first potential site and have the next-to-last site. Genotype D isolates all have the opposite pattern. They have the first potential glycosylation site but are missing the next-to-last site.

Antigenic variation in HPIV-1. The extent and type of antigenic variation that exists within an epidemic of HPIV-1 were determined by using 38 MAbs in ELISAs and HI assays against 15 clinical isolates. Twenty-two of the MAbs were directed against the HN protein, 2 MAbs were directed against the fusion (F) protein, 1 MAb was directed against the phosphoprotein (P), and 13 were directed against the nucleoprotein (NP). Twenty-two of the MAbs were made against the 1991 type strain (Mil-58/91 [genotype D]) and were selected because of their strong reactivity in ELISAs or microneutralization assays (Tables 1 and 2). Sixteen MAbs were made against the 1957 type strain (DC/57 [genotype A]) and were selected because they demonstrated the greatest amount of antigenic variation in previously tested isolates (7). Eight of the 22 1991 MAbs had significantly lower titers to the 1957 type strain. Likewise, 6 of 16 1957 MAbs demonstrated poor reactivity to the 1991 type strain.

The high specificities of the 11 HN 1957 MAbs have been published previously (7). The 11 1991 HN MAbs demonstrated similar specificities when tested in ELISAs against HPIV-2, -3, and -4 and Sendai virus (mouse PIV-1). Only 2 of 11 HN MAbs showed cross-reactivity with Sendai virus (Table 3). As expected, the NP MAbs showed more cross-reactivity, with 8 of 11 cross-reacting with Sendai virus and 1 of 11 cross-reacting with HPIV-3. Of the 22 HN-specific MAbs, 20 were neutralizing (Table 2).

HN protein demonstrates genotype-specific antigenic differences. Twenty-two HN MAbs were reacted with the clinical isolates in ELISAs. Of the 22 HN MAbs, 10 bound well to all of the HPIV-1 strains (MAbs HN57-18, HN57-30, HN57-44, HN57-31, HN57-37, HN91-44, HN91-60.2, HN91-67, HN91-78, and HN91-79.2). Four MAbs (HN91-37, HN91-56.2, HN91-61, and HN91-82) were specific for the 1991 isolates and did not react with the 1957 type strain. MAb (HN57-22) demonstrated fourfold-lower titers to three genotype C isolates. One 1991 MAb (HN91-38) and one 1957 MAb (HN57-43) were each specific to the strain that they were made against (Table 1). Four MAbs (HN57-11, HN57-24, HN57-39, and HN57-36) bound well to the previously described genotype A isolates (Table 1). These four MAbs did not bind well to the

TABLE 1. Reactivities of representative MAbs against HPIV-1 proteins to 15 clinical isolates by two assays[a]

MAb	Assay	Titer[b] of type strain		Result for[c]:											
				Genotype C clinical isolate						Genotype D clinical isolate					
		DC-1957	Mil-1991	48/91	49/91	50/91	51/91	56/91	60/91	63/91	64/91	52/91	53/91	54/91	55/91	58/91	61/91	62/91
HN57-11	ELISA	1M	<1K	++	++	++	++	++	++	++	++	+	+	+	+	+	+	+
	HI	384	16	−2.5	++	++	++	++	−3.5	−3.5	−3	++	++	++	++	++	−3.5	++
HN57-24	ELISA	12M	<10K	++	+	+	++	+	+	+	+	+	+	+	+	+	+	+
	HI	512	<16	++	++	++	−3.5	++	++	++	++	++	++	++	++	++	++	++
HN57-39	ELISA	800K	<1K	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	HI	192	<16	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
HN57-36	ELISA	2M	<1K	++	++	++	++	+	+	++	++	+	++	+	+	+	+	+
	HI	16	<16	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
HN57-43	ELISA	2M	<1K	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	HI	256	<16	++	++	++	++	++	++	++	−3	++	++	++	++	++	++	++
HN91-38	ELISA	<800K	23M	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	HI	<16	16	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
HN91-53.2	ELISA	700K	10M	−3	++	−3	−3	++	++	−3	−2	++++	++++	++++	++++	++++	++++	++++
	HI	24	192	++++	−2.5	−2	++++	++++	++++	−2.5	++++	++++	++++	++++	−2	++++	2	++++
F57-32	ELISA	2M	1M	−2	−2	−2	−2	−3	++	++++	++++	−2	3	++++	−2	++++	++++	++++
P57-28	ELISA	800K	8K	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
NP91-41	ELISA	5K	2M	−3	++	−2	++++	++	++	−3	−2	−2	−2	++	−2	++++	++++	++++

[a] Representative MAbs to HPIV-1 1957 type strain and HPIV-1 1991 strain (58/91) HN, fusion protein (F), phosphoprotein (P), and nucleoprotein (NP) titered in ELISAs and HI assays to 15 clinical isolates collected during the 1991 epidemic.

[b] Titers determined by ELISAs are reported as reciprocal values in thousands (K) or millions (M). Titers determined by HI assays are reported as reciprocal values.

[c] Data are exponential differences (base 2) from the immunizing strain used to produce the MAbs. Symbols: ++++, differences of <4-fold from the value for type strain; ++, differences of ≥16 fold lower than the value for the type strain; +, differences of ≥16 fold lower than the values for the other Milwaukee isolates. N, MAbs demonstrating no HI activity.

TABLE 2. Microneutralization titers of representative MAbs in reaction with different HPIV-1 genotypes

MAb	Microneutralization titer[a]			
	Genotype A		Mil-49/91 (genotype C)	Mil-58/91 (genotype D)
	1957 Type strain	CH/19-80		
HN57-43	>10,240	ND	ND	320
HN91-37	320	320	>10,240	>10,240
HN91-53.2	320	640	640	>10,240
HN91-56.2	2560	ND	ND	>10,240
HN91-61	320	320	>10,240	ND

[a] Titers reported as reciprocal values. ND, not done.

1991 HPIV-1 isolates, indicating that these MAbs continue to define a unique genotype. However, one of these MAbs (HN57-11) bound significantly better (≥16-fold) to 8 of the 15 clinical isolates (genotype C [Table 1]). Another of these MAbs (HN57-36) bound significantly better to six of eight of these same isolates. Finally, MAb HN91-53.2 bound better to the seven strains of HPIV-1 not bound by HN57-11 (genotype D [Table 1]). MAb HN91-53.2 demonstrated greater than eightfold-lower titers against seven of the eight subgroup C strains than against the subgroup D strains.

HI assays were performed to look for possible variation in the functional properties of the HN proteins (erythrocyte binding) instead of only structural changes as detected by ELISAs. It has been shown that these two properties are not always linked (7). HI titers ranged from <16 to >1,024. In general, the HI data paralleled the ELISA data, but MAb HN91-53.2 had less than a fourfold drop in HI assay for five of eight of the genotype C isolates. These same isolates had greater than eightfold (two were >16-fold) drops in their ELISA titers to HN91-53.2. This might simply reflect the relatively low HI titer to the type strain itself, even though this MAb is strongly neutralizing. MAbs HN91-61 and HN91-82 showed decreased HI titers to clinical isolates 49/91 and 51/91 without concomitant decrease in their ELISA titers.

Microneutralization confirms antigenic differences of genotypes. Five MAbs were tested in microneutralization assays against representative strains of genotypes A, C, and D (Table 2). Genotype-specific neutralization was clearly demonstrated. MAb HN91-53.2 easily differentiated genotype D from genotype A or C.

Variation detected in other structural proteins. Only two MAbs against the F protein were studied. MAb F57-32 showed significant variations in binding for the different isolates (Table 1). Three strains had 8- to 16-fold drops in MAb binding, while six isolates had a 4-fold decline. Six clinical strains had no significant change in titer or had increased binding. The difference in binding between strains 53/91 and 54/91 was >128-fold. The other F MAb (F57-12) demonstrated no variation between isolates. The one P MAb did not bind well to any of the 1991 isolates (Table 1). This was not unexpected, since this MAb helped define the genotype A strains. The F-protein antigenic variation was not genotype specific.

Thirteen MAbs directed against the NP protein were analyzed. They demonstrated less heterogeneity. Nine of these MAbs bound well to all HPIV-1 isolates (NP57-2, NP57-14, NP91-5, NP91-8, NP91-26, NP91-33, NP91-47, NP91-52, and NP91-66). One MAb (NP91-84) did not bind well to the 1957 type strain but bound well to all the 1991 strains. Two MAbs (NP91-30 and NP91-36) did not react as well in ELISAs against three clinical isolates (60/91, 56/91, and 48/91). The last NP MAb (NP91-41) did not bind well to the majority of the 1991 strains tested or to the 1957 type strain (Table 1). This poor binding was evident in both genotypes.

Evolutionary tree of HPIV-1 epidemic strains. The 13 HN nucleotide sequences from the 1991 Milwaukee epidemic were compared to find the minimum evolutionary tree for this set of clinical isolates. All methods yielded similar trees that varied only slightly in their terminal branches. Figure 2 is a representative tree for the 1991 epidemic. This tree was left unrooted because the immediate ancestor is unknown. The 1957 type strain (genotype A) is placed on the tree for orientation. The two antigenic and genetic subgroups (genotypes C and D) that have been previously described form two clear branches. This tree suggests that the immediate ancestor for each of the two genotypes came to Milwaukee and then further evolved into the variants within each genotype. The actual time frames for these mutations are unknown; whether other genotypes cocirculate during different epidemics is also not known.

Clinical disease. We have previously reported in detail the clinical epidemiology of the different HPIV-1 isolates (12). No

TABLE 3. Binding of representative 1991 MAbs to different viruses in ELISAs[a]

MAb	HPIV-1D[b]	HPIV-1A[c]	HPIV-2	HPIV-3	HPIV-4	Sendai virus[d]	No antigen (control)
HN-53.2	++++	+	−	−	−	−	−
HN-61	+++	−	−	−	−	−	−
HN-67	++	++	−	−	−	−	−
HN-78	+++	+++	−	−	−	++	−
HN-79	+++	++	−	−	−	+++	−
HN-82	+++	−	−	−	−	−	−
NP-5	+++	++++	−	−	−	−	−
NP-8	++++	++++	−	−	−	+++	−
NP-30	++	++	−	+	−	+++	−
NP-36	+	−	−	−	−	+	−
NP-52	+++	++++	−	−	−	+++	−
NP-84	+++	+	−	−	−	−	−

[a] ELISA was performed with an antigen concentration of 1 μg per well. Data are averages of two experiments where the optical density (OD) (A_{405}) was >0.100 and 3 times more than the value for the well with no antigen to be positive (+). Symbols: +, OD of 0.100 to 0.300; ++, OD of 0.300 to 0.600; +++, OD of 0.600 to 0.900; ++++, OD >0.900.

[b] HPIV-1D, HPIV-1 genotype D.

[c] HPIV-1A, HPIV-1 genotype A.

[d] Mouse PIV-1.

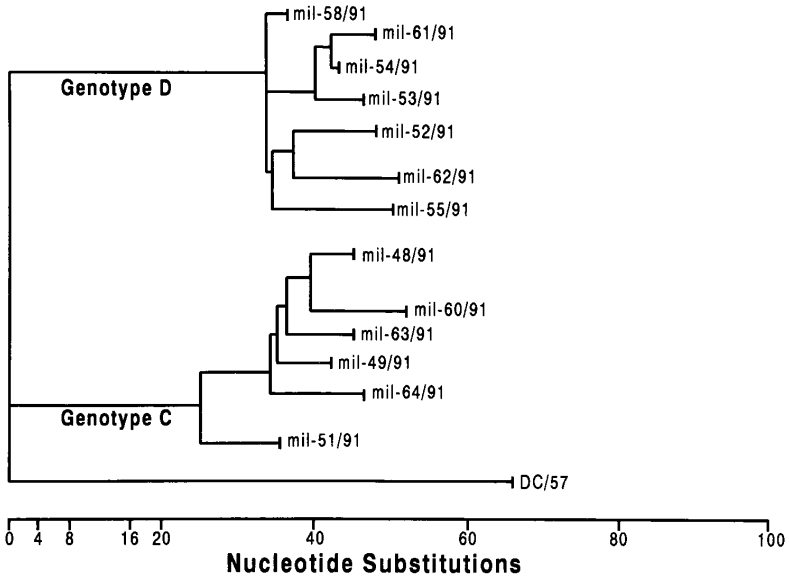

FIG. 2. Evolutionary tree for HN genes from HPIV-1 isolated during the 1991 Milwaukee epidemic. The length of the horizontal bar is proportional to the number of nucleotide substitutions. The tree is unrooted. The 1957 type strain (genotype A) is included for comparison.

subgroup-specific differences were found in either disease presentation or severity.

DISCUSSION

We analyzed 15 HPIV-1 clinical isolates to determine the extent of antigenic and genetic variation within the viral population from a single geographic location during a single epidemic. Previous work has examined only one or two viruses from any 1 year (7, 14, 15, 17). This information could be important to help determine which HPIV-1 antigens are needed in vaccines and whether more than one strain is needed in future vaccines and to help understand the factors leading to biennial epidemics.

Using a panel of MAbs against both the 1957 type strain and against an isolate from the 1991 epidemic, we showed that two antigenically distinct populations of HPIV-1 circulated equally during the 1991 epidemic in Milwaukee. The antigenic differences detected between genotypes C and D using our current panel of MAbs were few but corresponded with our genetic analysis. Two MAbs (HN57-11 and HN57-36) identified genotype C isolates and one (HN91-53.2) identified genotype D. Furthermore, these two genotypes were antigenically distinct from our previously described genotype A viruses (7). The epitopes corresponding to MAbs HN57-11, HN57-24, HN57-36, and HN57-39 are all strongly present on HPIV-1 genotype A strains but were reported to be absent in Texas isolates (15) and are either undetectable (MAbs HN57-24 and HN57-39) or significantly altered (MAbs HN57-11 and HN57-36) in Milwaukee strains.

The MAbs identifying genotypes A, C, and D are all (except MAb HN57-36) highly neutralizing. MAb HN91-53.2 neutralizes genotype D strains but not genotype A or C strains. Likewise, MAb HN57-43 neutralizes the 1957 type strain but not subgroup D viruses. These results suggest that these antigenic differences represent dominant neutralizing epitopes that may have been selected by restricted antibody responses in children (27).

Previously, we demonstrated that HPIV-1 clinical isolates collected over several decades could be subgrouped antigenically and genetically (7, 14). Subgroup A viruses had distinct MAb binding; all the rest of the isolates (called B [other] subgroup) had no specific MAb binding unique to them as a group. Genetic analysis confirmed that viruses categorized as A were different from those categorized as B. Genotype A demonstrated 28 unique nucleotide substitutions and eight amino acid substitutions. The B subgroup of HPIV-1 had 18 unique nucleotide substitutions and seven amino acid substitutions but demonstrated much more interisolate variation. For convenience, we talked about subgroup B but now that we have defined genotypes C and D, clearly the isolates in the B category are not as closely related as genotypes A, C, and D and have no specific antigenic characteristics.

Antigenic variation and change within HPIV-1 may be partially dependent on differences in N-linked glycosylation (23). Previous work demonstrated significant variability in the number of potential N-linked glycosylation sites on the HN proteins of different clinical isolates (14). Genotype C and D strains demonstrated consistently different patterns of potential N-linked glycosylation. The significance of these observed patterns has not been determined.

The presence of two distinct genotypes with minor antigenic differences during one outbreak suggests that geography may not be the current evolutionary force leading to the continuation of these genotypes (15). Instead, multiple unique genotypes may circulate throughout this country and then reappear when they experience immunologic pressure to form multiple quasispecies around the evolving parent genotypic strain. Also, different parts of the HPIV-1 genome may be under different evolutionary pressures (1). Sequence analysis of other HPIV-1 genes will help clarify the evolution of this virus.

The closely related respiratory syncytial virus (RSV) has demonstrated two major antigenic groups (groups A and B) mostly by antigenic and genetic differences found in their surface glycoproteins (G and F) (2, 3). Group A viruses have been reported to be more virulent than group B strains (19). Evidence suggests that children's immune responses and protection against infection and reinfection are group specific and that vaccine strategies need to include both group A and B strains of RSV (20). The genetic differences between RSV

groups A and B are much larger than what we find between HPIV-1 genotypes C and D (16). However, subgroups of RSV group A have been described that are similar to the genotypes that we have described (22). No information is available about the biologic significance of these RSV subgroups.

Viruses even more closely related to HPIV-1 (HPIV-2, -3, and -4) have been shown to have significant antigenic and genetic diversity and have been categorized into genotypes and subgroups (9, 18, 21, 25, 27). Recently HPIV antigenic diversity was underscored when a clinical isolate of HPIV-3 could not be detected with MAbs from two commercial sources (24). Understanding this variation may be important in producing broad protection in infants and children to these common respiratory pathogens. Furthermore, Sendai virus (mouse PIV-1) has been shown to have strains that can be subgrouped by differences in their virulence (29). This has also been suggested for HPIV-1 (26) and HPIV-3 (30).

Previously we demonstrated that HPIV-1 is antigenically and genetically heterologous with multiple circulating strains that appear and reappear over decades (7, 14). We also showed that HPIV-1 had at least one genotype defined by specific MAb binding and genetic analysis (genotype A). Children made genotype-specific HI antibody responses to HPIV-1 (7). Poor cross neutralization of heterologous genotypes during childhood reinfection with HPIV-1 could be part of the explanation for biennial epidemics.

We have now added to this knowledge by demonstrating that local epidemics contain at least two genotypes with specific antigenic characteristics circulating and causing clinical disease at the same time. Although geography may have originally led to the formation of HPIV-1 genotypes, local immunologic pressure may be maintaining them. MAbs can be genotype specific in their ability to neutralize HPIV-1. Genotypes C and D are unique from the genotype A strains previously described and form separate evolutionary branches. It is still not clear whether one genotype of HPIV-1 stimulates a broad enough antibody response in children to protect them against subsequent exposure to heterologous genotypes. A complete evolutionary and structural analysis of the HPIV-1 HN protein is under preparation.

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