JOURNAL OF VIROLOGY, Mar. 1990, p. 1329–1334
0022-538X/90/031329-06$02.00/0

Vol. 64, No. 3

Naturally Occurring Human Parainfluenza Type 3 Viruses Exhibit Divergence in Amino Acid Sequence of Their Fusion Protein Neutralization Epitopes and Cleavage Sites

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Many human parainfluenza type 3 virus (PIV3) strains isolated from children with respiratory illness are resistant to neutralization by monoclonal antibodies (MAbs) which recognize epitopes in antigenic site A or B of the fusion (F) protein of the prototype 1957 PIV3 strain. The F protein genes of seven PIV3 clinical isolates were sequenced to determine whether their neutralization-resistant phenotypes were associated with specific differences in amino acids which are recognized by neutralizing MAbs. Several clinical strains which were resistant to neutralization by site A or B MAbs had amino acid differences at residues 398 or 73, respectively. These specific changes undoubtedly account for the neutralization-resistant phenotype of these isolates, since identical substitutions at residues 398 or 73 in MAb-selected escape mutants confer resistance to neutralization by site A or B MAbs. The existence of identical changes in naturally occurring and MAb-selected neutralization-resistant PIV3 strains raises the possibility that antigenically different strains may arise by immune selection during replication in partially immune children. Three of the seven clinical strains examined had differences in their F protein cleavage site sequence. Whereas the prototype PIV3 strain has the cleavage site sequence Arg-Thr-Lys-Arg, one clinical isolate had the sequence Arg-Thr-Arg-Arg and two isolates had the sequence Arg-Thr-Glu-Arg. The different cleavage site sequences of these viruses did not affect their level of replication in either continuous simian or bovine kidney cell monolayers (in the presence or absence of exogenous trypsin or plasmin) or in the upper or lower respiratory tract of rhesus monkeys. We conclude that two nonconsecutive basic residues within the F protein cleavage site are sufficient for efficient replication of human PIV3 in primates.

Parainfluenza type 3 virus (PIV3) is an important respiratory tract pathogen of infants and children (2). Infection of host cells by PIV3 is initiated by two virion surface glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion (F) proteins. After the HN protein binds to sialic acid-containing host cell receptors, the F protein mediates fusion of the virion envelope with host cell membranes, thereby delivering virus nucleocapsids into the cytoplasm. The F protein is also responsible for fusion of infected cells with uninfected cells during later stages of replication, resulting in the appearance of syncytia in infected cell monolayers (21).

Antibodies to the HN or F protein are thought to mediate their antiviral effect by inhibiting virus attachment, penetration, or cell-to-cell spread of infection. Monoclonal antibodies (MAbs) which recognize six antigenic sites on the HN protein have been produced, and neutralizing MAbs recognize 11 epitopes which map to three of the six sites (6). Similarly, anti-F MAbs detect eight antigenic sites on the F protein and neutralizing MAbs recognize 14 epitopes within four of the antigenic sites (3). Antigenic variants of the prototype strain, selected in vitro in the presence of neutralizing anti-F or anti-HN MAbs, have sustained a single amino acid substitution in the F or HN protein which confers resistance to neutralization by MAbs (4, 5). Human PIV3 is a monotypic paramyxovirus, but antigenic polymorphism has been demonstrated in five of 11 HN neutralization epitopes of clinical PIV3 isolates (6). In contrast to the relative antigenic stability of the HN, only three of 14 neutralization epitopes of the F protein are conserved among all 37 human PIV3 strains examined and over half of these strains are refractory to neutralization by specific anti-F MAbs (3). To determine whether the neutralization-resistant phenotypes of these naturally occurring strains were associated with specific amino acid differences, we sequenced the F genes of seven clinical strains which are not neutralized by one or more MAbs. Comparison of the antigenic phenotypes and F gene sequences of the clinical isolates with those of the prototype PIV3 strain and of laboratory-selected variants revealed that the deduced F protein sequences of the clinical isolates differed very little (less than 3%) from that of the prototype strain. Significantly, however, the differences among clinical strains frequently involved the same amino acids which are substituted in the neutralization epitopes of laboratory-selected MAb-resistant variants.

In addition to having amino acid differences in their neutralization epitopes, three of the isolates also had differences in the proteolytic cleavage site sequence of the F protein. Proteolytic cleavage of the F protein into two disulfide-linked subunits (22) is necessary for paramyxovirus infectivity and fusion activity (21), and cleavability of the F has been shown to be an important determinant of pathogenicity and virulence for avian paramyxoviruses (11). For canine and avian paramyxoviruses, both the number and pairing of basic amino acids within the F protein cleavage site appears to influence the efficiency with which it is cleaved by trypsinlike host cell enzymes (11, 19). The prototype human PIV3 cleavage site consists of three basic residues, two of which are consecutive. The single amino

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acid difference in the cleavage site of one clinical strain maintained the prototype number and pairing of basic residues. The cleavage site sequence of two other clinical isolates differed at two positions and had only two nonconsecutive basic residues. To determine whether changes in the cleavage site were associated with altered growth properties, we compared the replication of the three cleavage site variants and the prototype PIV3 strain in cell monolayers, with or without the addition of exogenous proteases, and in the upper and lower respiratory tract of rhesus monkeys.

MATERIALS AND METHODS

Cells and viruses. LLC-MK2 monkey kidney and Madin-Darby bovine kidney (MDBK) cells were grown in Eagle minimum essential medium supplemented with 10% fetal bovine serum. The prototype PIV3/Washington/47885/57 strain was obtained from the Research Resources Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Virus strains Washington/1511/73, Washington/S6543/73, Washington/589/75, Texas/545/80, Texas/535/81, Texas/6166/82, and Texas/12677/83 were isolated from children with acute respiratory illness and were obtained from Barbara Jeffries (National Children's Hospital Medical Center, Washington, D.C.) and Julius Kasel (Baylor University, Houston, Texas). The reactivities of these viruses in enzyme-linked immunosorbent assay and neutralization assays with MAbs produced to the F protein of the prototype PIV3 have been described previously (3). For the present study, these virus isolates were plaque purified in LLC-MK2 cell monolayers and their reactivity patterns with the panel of MAbs were confirmed. Semiconfluent LLC-MK2 cell monolayers were infected with PIV3 at a multiplicity of 1.0 in serum-free Eagle minimum essential medium maintenance medium. For in vitro virus replication experiments, trypsin (1 μg/ml; Worthington Diagnostics, Freehold, N.J.) or plasmin (5 μg/ml; Calbiochem-Behring, La Jolla, Calif.) was added to the maintenance medium. For production of purified virions, virions were pelleted from the medium 72 h postinoculation and were purified by two cycles of sedimentation through 30% sucrose onto a cushion of 45% sucrose.

Nucleic acid sequence analysis. Sequence analysis of the F gene PIV3 clinical isolates was performed by using a modification of the Sanger method. Briefly, synthetic oligonucleotide primers, based on the published PIV3 F gene sequence (23), were used to sequence genomic RNA by reverse transcription in the presence of dideoxynucleotides as previously described (7). Ambiguities in the sequence resulting from premature termination of reverse transcription were resolved by extending the premature termination products of the original sequencing reactions with terminal deoxynucleotidyl transferase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) by using the method of DeBorde et al. (10). Of the 14.8 kilobases of RNA sequenced in this study, there were 50 bases which could not be resolved by using these methods. Of the ambiguous bases, 30 were located in the 5′ untranslated region and 20 were located in the coding region of the F gene. These 20 ambiguous bases in the coding region prevented unequivocal identification of amino acid 37 of Texas/6166/82, amino acids 104, 105, 224, 228, and 335 of Washington/S6543/73, amino acids 403 and 481 of Texas/535/81, and amino acid 230 of Texas/545/80.

Virus replication in rhesus monkeys. Replication of PIV3 clinical strains was compared in rhesus monkeys. Groups of four rhesus monkeys were inoculated intratracheally with 10^5 50% tissue culture infectious doses of infectious virus.

TABLE 1. Distribution and frequency of nucleotide and amino acid differences[a] in PIV3 genes and proteins

F gene from indicated strain	Nucleotide differences in the:			Amino acid differences (%)
	Noncoding region		Coding region	
	5′ Noncoding 1 to 193[b]	3′ Noncoding 1811 to 1845[b]	194 to 1810[b]	
Wash/1511/73	24 (12.4)	2 (5.7)	58 (3.6)	9 (1.7)
Wash/S6543/73	25 (12.9)	1 (2.9)	60 (3.7)	9 (1.7)
Wash/589/75	ND[c]	ND	50 (3.0)	8 (1.5)
Tex/545/80	28 (14.5)	3 (8.5)	63 (3.9)	11 (2.0)
Tex/535/81	27 (13.9)	3 (8.5)	64 (3.9)	13 (2.4)
Tex/6166/82	33 (17.1)	1 (2.9)	66 (4.1)	11 (2.0)
Tex/12677/83	29 (15.0)	3 (8.5)	69 (4.3)	13 (2.4)
% Mean variability	14.3	6.2	3.8	2.0

[a] Percent differences were calculated with respect to the prototype Washington/47885/57 strain.
[b] Nucleotides defining regions of the F gene.
[c] ND, Not done.

Nasopharyngeal swab specimens were obtained from monkeys daily for 12 days after inoculation, and tracheal lavage specimens were obtained on days 2, 4, 6, and 8 after inoculation. Specimens were stored frozen at −70°C, and the amount of virus present in nasopharyngeal swabs and tracheal lavage specimens was determined by inoculating 20 μl of decimal dilutions of test material onto LLC-MK2 cell monolayers in 96-well plates. The monolayers were observed for cytopathic effects, and virus replication was confirmed by hemadsorption of cells with 0.1% guinea pig erythrocytes.

RESULTS

Nucleotide and deduced amino acid sequences of PIV3 isolates. The F gene nucleotide sequences of seven clinical PIV3 strains were determined and compared with the F gene sequences of the prototype PIV3/Washington/47885/57 strain. The PIV3 F gene is transcribed into a message of 1,845 nucleotides, which has a coding region of 1,617 nucleotides and 5′ and 3′ noncoding regions of 193 and 35 nucleotides, respectively (23). The F genes analyzed contained no deletions or insertions and were approximately 95% conserved in overall sequence. The low frequency of nucleotide differences in the 3′ noncoding region and in the coding region of the F genes (6.2% and 3.9%, respectively) approximated the conservation of the corresponding regions of the PIV3 HN genes (5) (Table 1). Most of the F gene sequence variability occurred in the 5′ noncoding region (14.3% mean variability), which is also the most divergent region of the HN genes of PIV3 strains (6.8% mean variability).

The predicted amino acid sequences of the F proteins were highly conserved (Table 1). All of the cysteines and potential N-linked glycosylation acceptor sites were conserved among the clinical strains, and of the 539 F amino acids, only 20 differences were identified among the seven F proteins compared. Of the 20 amino acids which differed from the prototype sequences, 5 were the same in all seven strains (Fig. 2) and, therefore, probably represent the PIV3 F

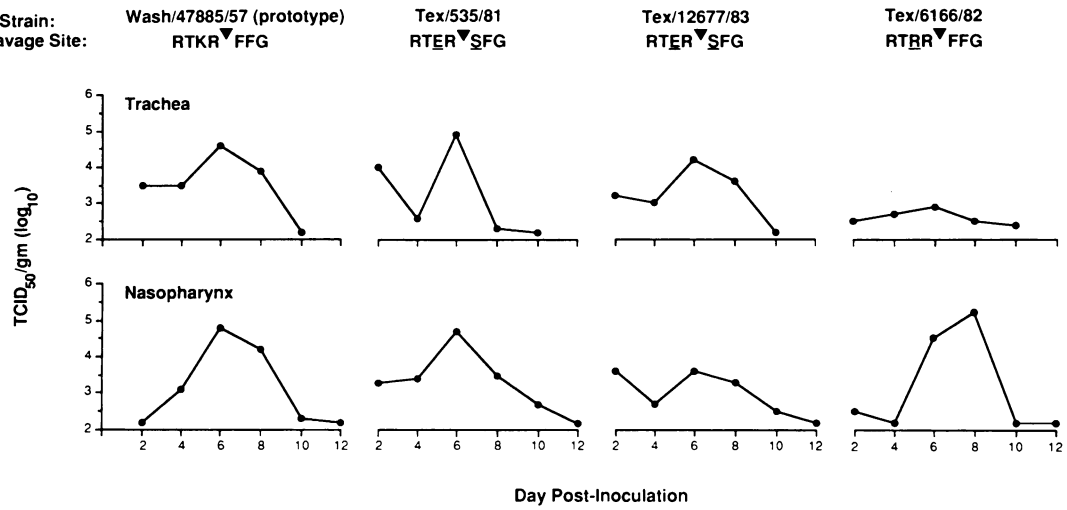

FIG. 1. Replication of prototype PIV3 (Washington/47885/57) and cleavage site variants (Texas/6166/82, Texas/12677/83, and Texas/535/81) in rhesus monkeys. The amino acid sequence at positions 106 to 112 is shown for each strain. The arrow marks the site of F protein cleavage (23), and differences from the prototype sequence are underlined. Groups of four monkeys were inoculated intratracheally with 10^5 50% tissue culture infective doses of virus. The amount of virus present in tracheal lavage (upper panels) and nasopharyngeal swab (lower panels) specimens was quantitated as described in Materials and Methods.

consensus sequence in these positions. The remaining 14 variable amino acids represent less than 3% of the F protein amino acids.

Identification of cleavage site variants and analysis of their replication in vitro and in vivo. The F protein cleavage site sequence of three of the seven PIV3 clinical strains examined was different from that of the prototype PIV3 strain (Fig. 1). The prototype human PIV3 cleavage site sequence consists of three basic residues, two of which are consecutive (Arg-106-Thr-Lys-Arg-Phe-110). Cleavage at the Arg-Phe bond by a host cell trypsinlike enzyme generates the two disulfide-linked subunits of the F protein. The consecutive difference (Arg-108 instead of Lys) within the cleavage site sequence of Texas/6166/82 did not change the number or pairing of basic residues in the cleavage site. Texas/12677/83 and Texas/535/81 each had two differences: Glu-108 rather than Lys, which changed the number and pairing of basic residues in the cleavage site, and Ser-110 rather than Phe at the cleavage site. Since other paramyxoviruses require a minimum number of consecutive basic residues within the cleavage site for efficient cleavage (11, 19, 25) and virulence (11, 25), it was of interest to determine whether the observed changes within the cleavage site of some PIV3 strains was associated in vitro or in vivo with altered replication. The cleavage site variants and the prototype strain did not require the addition of exogenous trypsin or plasmin for replication in either LLC-MK2 monkey kidney or MDBK bovine kidney cell monolayers, nor was the amount of infectious virus produced in these cell lines increased by the addition of exogenous trypsin or plasmin (data not shown), suggesting that the above-described cleavage site alterations did not affect the cleavability of the F protein in these cell lines.

To determine whether the cleavage site differences in the clinical isolates were associated with altered replication in vivo, we compared their replication in rhesus monkeys. With one exception, the cleavage site variants replicated in the nasopharynx as well as the prototype strain, achieving mean peak virus titers of $10^{4.7}$ to $10^{5.2}$ 50% tissue culture infective doses per ml on days 6 to 8 (Fig. 1). Replication of Texas/12677/83 in the nasopharynx was slightly reduced compared with other strains evaluated. However, this reduction did not correlate with a specific cleavage site sequence: Texas/535/81 (which has the same cleavage site sequence as Texas/12677/83) replicated efficiently in the nasopharynx. In the trachea, all but one of the cleavage site variants replicated as well as the prototype strain, achieving mean peak virus titers of $10^{4.2}$ to $10^{4.9}$ 50% tissue culture infective doses per ml on day 6. The one exception, Texas/6166/82, was 50-fold restricted in its replication in the trachea but has the same number and pairing of basic residues in the cleavage site as the prototype strain. These results indicated that the cleavage site sequence differences found in the clinical isolates sequenced do not affect their ability to replicate in the upper and lower respiratory tract of monkeys.

Identification of amino acid differences responsible for resistance of clinical strains to MAb neutralization. We have previously shown that the F proteins of many PIV3 clinical strains undergo antigenic variation which can be detected by using MAbs in neutralization assays (3). Laboratory variants selected with MAbs have specific amino acid substitutions in F protein neutralization epitopes which are responsible for their resistance to neutralization by MAbs (4). To determine whether differences in the same amino acids are responsible for the neutralization-resistant phenotypes of naturally occurring PIV3 strains, we compared the deduced amino acid sequences of the F proteins of seven PIV3 clinical isolates with that of the prototype PIV3 strain and of laboratory-selected variants derived from it.

The F proteins of the clinical strains were remarkably similar to the prototype PIV3 strain: of 539 amino acids, only 20 amino acid differences were identified among the seven F proteins compared (Fig. 2). This level of conservation was somewhat unexpected, since the seven strains were selected from a total of 37 strains because they were resistant to neutralization by one or more MAbs (3). Of the 20 differences, 2 were located in the signal peptide, which is removed from the mature F protein and would not, therefore, contribute to antigenic differences among the PIV3 strains. Furthermore, 4 of the 20 differences were located in the

NEUTRALIZING ANTIBODIES:				PIV3 STRAINS:								
MAB	ANTIGENIC SITE	EPITOPE	POSITION OF AMINO ACID CHANGE IN VARIANTS SELECTED WITH MABS	Wash/47885/57 (prototype) *	Wash/1511/73 *	Tex/6166/82	Wash/S6543/73	Tex/545/80	Tex/535/81	Wash/589/75 *	Tex/12677/83 *
a197		N1	398	+	+	+	O	O	O	+	+
c267		N2	397	+	O	O	O	O	O	O	+
c214		N3	396	+	+	O	O	O	O	O	O
c279	A	N4	396	+	+	O	O	O	O	+	+
c284		N5	N.D.	+	+	O	O	O	O	+	+
c199		N6	N.D.	+	+	+	O	O	O	+	+
c191		N7	397	+	+	+	O	O	O	+	+
b102		N8	37	+	+	O	+	+	+	+	+
c145	AB	N9	N.D.	+	+	+	+	+	+	+	+
c108		N10	149	+	+	+	+	+	+	+	+
c128		N11	73, 452	+	+	+	+	O	O	O	O
c110	B	N12	73	+	+	+	+	O	O	O	O
c215		N13	73	+	+	+	+	O	O	O	O
a640	C	N14	287	+	+	+	+	+	+	+	+
			SIGNAL 8	I					L		
			SIGNAL 17	F		S					
			73	R				K	K	K	K
			F_2 95	S	T	T	T	T	T		T
			101	E							D
AMINO ACID AT INDICATED POSITION:			108	K		R			E		E
			110	F					S		S
			141	R		K					
			•295	R	K	K	K	K	K	K	K
			•367	V	T	T	T	T	T	T	T
			•369	K	T	T	T	T	T	T	T
			398	G			D	D	D		
			409	V				I	I		I
			F_1 418	N	S	S	S	S	S		S
			441	N	G						
			492	T		I					
			ANCHOR 497	V	I		I	I		I	I
			ANCHOR 509	V						I	
			ANCHOR •513	I	T	T	T	T	T	T	T
			ANCHOR •516	V	T	T	T	T	T	T	T

FIG. 2. Antigenic phenotypes and amino acid differences in the F protein of seven PIV3 clinical isolates. Neutralizing MAbs which recognize epitopes N1 through N14 in antigenic site A, AB, B, or C of the PIV3 F protein of the 1957 prototype strain, and the amino acid substitutions in laboratory variants which are selected with these MAbs are shown. Clinical PIV3 isolates sequenced without ambiguity are indicated (*), and their susceptibility (+) or resistance (O) to neutralization by individual MAbs is indicated. Amino acids which differ from the prototype sequence are shown for each strain, and differences which represent the PIV3 F consensus sequence (see Results) are indicated (•). Boxes highlight residues which are also substituted in MAb-selected laboratory variants (4).

membrane anchor, where they would be unlikely to influence antibody recognition. Of the 20 changes in the F proteins of the clinical strains, therefore, only 14 involved amino acids in the ectodomain of the mature F protein. Within the ectodomain, 5 of the 14 amino acid differences (at positions 295, 367, 369, 513, and 516) represented the consensus sequence of the F proteins analyzed (see Fig. 2), and could not, therefore, account for the different neutralization patterns. The 11 remaining differences in the 475-residue ectodomain of the mature F protein represented variability which was unique to different isolates and could, therefore, be responsible for their unique antigenic phenotypes.

We have previously shown that antigenic site A of the PIV3 F protein consists of eight overlapping neutralization epitopes. Recognition of these epitopes by site A MAbs is abrogated by a substitution at amino acid 37, 396, 397, or 398 in laboratory-selected neutralization-resistant variants (4). All of the clinical strains analyzed in the present report were resistant to neutralization by one or more site A MAbs (Fig. 2). Sequence analysis indicated that three of these strains (Washington/S6543/73, Texas/545/80, and Texas/535/80) have an Asp at position 398, which is Gly in the prototype sequence. Although several amino acids in these viruses could not be unequivocally identified (see Materials and Methods), the amino acid difference at position 398 (which is the only difference shared by the three strains) probably accounts for the resistance of these three strains to neutralization by seven of the site A MAbs, since a single substitution of Asp for Gly-398 in laboratory-selected variants has been shown to confer resistance to these MAbs (4).

Three of the seven clinical strains which were resistant to neutralization by one, two, or five site A MAbs (Washington/1511/73, Washington/589/75, and Texas/12677/83) (Fig. 2) were sequenced without ambiguity and did not have alterations in any of the residues previously identified as critical for neutralization by site A MAbs (i.e., amino acids 37, 396, 397, or 398). However, each of them contained a unique difference which could account for their characteristic neutralization-resistant phenotype with the site A MAbs. For example, Washington/1511/73 was not neutralized by MAb c267 and had a unique change at position 441. Similarly, the resistance of Texas/12677/83 to MAb c214 could be due to a change at amino acid 101. Interestingly, the only change in the F protein of strain Washington/589/75 which could account for its resistance to site A MAbs c267 and c214 is at position 509 within the membrane anchor. The resistance of Texas/6166/82 to five site A MAbs might result from changes at residues 108, 141, and/or 492 but also could be due to a change in amino acid 37 (which could not be unambiguously deduced because of a sequencing artifact).

Antigenic site B of the PIV3 F protein is composed of three overlapping neutralization epitopes (N11 through N13) (Fig. 2). Recognition of these epitopes by site B MAbs is abrogated by a substitution at amino acid 73 in laboratory-selected neutralization-resistant variants (4). Furthermore, the type of substitution at position 73 and the presence of a second substitution at position 452 in these variants affect the ability of several site A MAbs to neutralize as well (4). Sequence analysis indicated that four of the clinical PIV3 strains which are resistant to neutralization by site B MAbs have a Lys at position 73, which is Arg in the prototype sequence. The difference at residue 73 is very likely to be responsible for the resistance of these four strains to the site B MAbs, because Lys-73 is the only difference shared by these strains, and a single substitution of Lys for Arg-73 in laboratory-selected variants confers resistance to these MAbs (4). Two of these strains which are resistant to site B MAbs (Texas/545/80 and Texas/535/80) are also resistant to neutralization by many site A MAbs, which, as described above, could be accounted for by a change in amino acid 398. The inability of one or two site A MAbs to neutralize Washington/589/75 and Texas/12677/83 cannot be associated with any specific amino acid differences in their F proteins but could be due to their respective differences at positions 509 or 37, 101, 108, and 409.

DISCUSSION

Nucleotide sequence analysis of seven PIV3 clinical isolated indicated that their F genes were highly conserved: compared with the prototype PIV 3 strain, the F genes differ by only 4.5 to 5.5%, the highest concentration of nucleotide divergence being in the 5′ untranslated region. The coding sequences of the F genes have greater than 96% nucleotide identity to the prototype strain, and the deduced amino acid sequences are more than 97% conserved among the strains. This level of amino acid conservation is higher than that of the hemagglutinin (HA) and neuraminidase proteins of influenza A viruses of the same antigenic subtype (9, 25).

Three of the clinical PIV3 isolates examined had cleavage site sequences which differed from that of the prototype PIV3 strain. Two of the strains had a Lys to Glu change at residue 108, which reduced both the number and pairing of basic residues within the cleavage site. A third PIV3 isolate had a Lys to Arg change at position 108, which maintained the number and pairing of basic residues of the prototype sequence. Changes in the cleavage sites of other paramyxoviruses influence the cleavability of the F protein (11, 19, 25) and, consequently, virus infectivity (21), but the PIV3 cleavage site variants were indistinguishable from the prototype strain in their level of replication in either bovine or simian kidney cell monolayers, and no correlation was found between the presence of two rather than three basic residues in the cleavage site and the level of replication in the upper or lower respiratory tract of rhesus monkeys.

These results indicated that two unpaired basic residues within the cleavage site are sufficient for cleavage and activation of the human PIV3 F protein in LLC-MK2 and MDBK cells and in the respiratory tract of primates. This stands in contrast to the F protein of simian virus 5, which requires a minimum of four basic residues for cleavage in LLC-MK2 cells (19), and Newcastle disease virus, which requires two pairs of consecutive basic residues in the cleavage site for cleavability and virulence (11, 25). On the other hand, both Sendai virus F and PIV1 have a single Arg in the cleavage site (14, 17) and both viruses require trypsin for growth in bovine or monkey kidney cells, respectively (12, 15). Also, structural features in addition to the number of pairs of basic amino acids influence whether trypsinlike host cell proteases (13) cleave paramyxovirus F proteins efficiently. The importance of structural elements other than the number of basic residues in the cleavage site has been demonstrated to modulate cleavability of the influenza A virus HA. These features include carbohydrate modification of amino acids proximal to the cleavage site (16) and changes in amino acids which are juxtaposed to the cleavage site in the folded HA molecule (20). The dramatic increase in cleavability of a pantropic Sendai virus variant which has a conservative (Arg to Lys) change in the cleavage site accompanied by a nonconservative change from Ser to Pro immediately upstream suggests that structural features in addition to basic amino acids within the cleavage site can also play a major role in ease of cleavage of the F protein (24). In addition, the acquisition of susceptibility to plasmin cleavage which is conferred by a Phe to Ser change immediately following the Sendai virus cleavage site suggests that regions of the F protein contiguous to the cleavage site can also affect cleavage (14). However, in this study the Phe to Ser change immediately following the cleavage site in two of our human PIV3 cleavage site variants did not appear to affect ease of cleavage.

We have previously documented that PIV3 strains having antigenically distinct HN and F proteins cocirculate in the human population and that their evolution is different from that of influenza A virus in that progressive accumulation of antigenic changes in PIV3 surface glycoprotein neutralization epitopes does not occur (3, 4). Results of the present study indicate that although PIV3 clinical isolates have almost identical degrees of amino acid variability in their HN and F proteins (about 3%), the locus of the variability is different. Whereas none of the HN proteins of clinical isolates sequenced had differences in amino acid residues known to be important for recognition by neutralizing anti-HN MAbs (7), many of the F proteins of clinical strains have amino acid differences which are identical to the substitutions found in laboratory variants which are selected by and resistant to neutralizing anti-F MAbs (4). Immune selection of neutralization-resistant variants is the mechanism which drives antigenic drift of influenza A (and possibly influenza B) viruses in the human population, which can be mimicked in the laboratory by selection of antigenic variants by using neutralizing anti-HA MAbs (27). For both influenza virus and PIV3, naturally occurring strains frequently have changes in more than one antigenic site of the HA or F, respectively, whereas laboratory-selected variants generally have a change in only one antigenic site (1, 3). This similarity suggests the possibility that the variable F epitopes of PIV3 clinical isolates might arise by immune selection and raises the additional question of whether this variation could play a role in the ability of PIV3 to reinfect children.

How could antigenic variation in F protein neutralization epitopes contribute to reinfection of children? It would seem that any possible replication advantage gained by strains with antigenically altered F neutralization epitopes would be abrogated by antibodies to highly conserved neutralization epitopes on both HN and F glycoproteins. Indeed, the ability of a few conserved HN and F neutralization epitopes to induce cross-protective immunity to antigenically divergent PIV3 strains has been previously demonstrated in rodents and monkeys (8). In contrast, it has been documented that many humans develop influenza A virus HA antibodies to a restricted number of epitopes (26) and that the sera of some children and adults can discriminate between two influenza A viruses which differ by only one amino acid in the HA (18). These studies, although they did not examine the ability of postinfection sera to neutralize antigenically different influenza A viruses, clearly demonstrated that individuals respond differently to infection by producing antibodies which may not recognize all or even a majority of protective epitopes. Therefore, it is possible that if primary infection of infants and children with PIV3 stimulates an F-specific antibody repertoire which is epitope-restricted (i.e., directed toward either nonneutralization epitopes or toward highly variable neutralization epitopes of the surface glycoproteins), strains having antigenically different F neutralization epitopes might have a selective advantage for infection of the partially immune host and, therefore, may be involved in individual cases of reinfection. Alternatively, if a subset of the F antibodies stimulated by primary infection are of short duration, strains having different antigenic phenotypes might have an advantage for reinfection of children. On the other hand, if children respond immunologically to the HN in a broad, antigenically unrestricted fashion, PIV3 strains having alterations in some HN epitopes would be neutralized by antibodies to conserved HN epitopes. Evaluation of the epidemiologic importance of antigenic variation in F neutralization epitopes will require a careful examination of the HN and F epitope-specific immune responses and the neutralizing antibody response in infants and children after primary infection and subsequent reinfection with PIV3.

ACKNOWLEDGMENTS

We thank B. Murphy and R. Chanock for helpful discussions and for critical reading of the manuscript, W. London and J. Phillips for inoculation and sampling of monkeys, D. Stec for help with the figures, M. Hill for synthesizing sequencing primers, and T. Heishman for editorial assistance.

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