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Structural Features Influencing Hemagglutinin Cleavability in a Human Influenza A Virus

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The cleavability of the hemagglutinin (HA) molecule is related to the virulence of avian influenza A viruses, but its influence on human influenza virus strains is unknown. Two structural features are involved in the cleavage of avian influenza A virus HAs: a series of basic amino acids at the cleavage site and an oligosaccharide side chain in the near vicinity. The importance of these properties in the cleavability of a human influenza A virus (A/Aichi/2/68) HA was investigated by using mutants that contained or lacked an oligosaccharide side chain and had either four or six basic amino acids. All mutants except the one that contains a single mutation at the glycosylation site were cleaved, although not completely, demonstrating that a series of basic amino acids confers susceptibility to cellular cleavage enzymes among human influenza virus HAs. The mutants containing six basic amino acids at the cleavage site showed limited polykaryon formation upon exposure to low pH, indicating that cleavage was adequate to impart fusion activity to the HA. Deletion of the potential glycosylation site had no effect on the cleavability of these mutants; hence, the oligosaccharide side chain appears to have no role in human influenza virus HA cleavage. The inability to induce high cleavability in a human influenza A virus HA by insertion of a series of basic amino acids at the cleavage site indicates that other, as yet unidentified structural features are needed to enhance the susceptibility of these HAs to cellular proteases.

Influenza A viruses representing 14 hemagglutinin (HA) and 9 neuraminidase subtypes have been isolated from birds and mammals. Most of these viruses replicate locally (i.e., in the intestinal tract in waterfowl and upper respiratory tract in mammals) and cause either no or limited disease symptoms unless complications are involved. However, some influenza A viruses of the H5 and H7 subtypes are highly pathogenic in domestic poultry, accounting for the deaths of large numbers of birds.

Although the virulence of influenza viruses is a polygenic trait (38), the HA plays an important role (3, 29). It is encoded by the fourth-largest RNA segment and is synthesized as a single polypeptide chain that undergoes posttranslational cleavage in at least two sites (for a review, see reference 21). An N-terminal signal sequence is removed and, depending on the host cell and virus strain, the molecule is cleaved with the removal of one or more intervening residues, or the connecting peptide, to yield two polypeptide chains, HA1 and HA2, with molecular weights of 36,000 and 27,000, respectively. Characterization of the three-dimensional structure of the A/Aichi/2/68 (H3N2) HA (45) has revealed a trimer composed of two structurally distinct regions: a triple-stranded coiled coil of α helix extending 7.6 nm from the membrane (the stalk) and a globular region of antiparallel β sheet containing the receptor-binding site. The cleavage site between HA1 and HA2 is located in the middle of the stalk. In tissue culture, the HA of virulent avian influenza viruses is cleaved into HA1 and HA2 in the absence of exogenous trypsin, whereas that of avirulent influenza viruses is not cleaved unless trypsin is added (3). Thus, the HAs of virulent and avirulent viruses have different sensitivities to endogenous cellular proteases.

Cleavage of the HA polypeptide into HA1 and HA2 is a requirement for infectivity (19, 22), because it generates the hydrophobic amino terminus of the HA2, which mediates fusion between the viral envelope and the plasma membrane (for a review, see reference 44). Therefore, the HAs of both virulent and avirulent viruses must be cleaved in vivo. Why the sensitivity of the HA to cellular proteases is correlated with virulence is unclear. One possibility could be the difference in tissue distribution of the cleavage enzymes. The enzymes capable of cleaving the HA of virulent viruses may be ubiquitous, allowing the viruses to replicate in many tissues, whereas those for the avirulent viruses may be limited to certain tissues, thus restricting virus replication.

As an initial step in understanding the correlation between viruses and the sensitivity of the HA to cellular enzymes, we established the requirements for cleavage of the avian influenza virus HA (16, 17). Two structural features, a series of basic amino acids at the cleavage site and an oligosaccharide side chain in the near vicinity, were found to be interrelated in determining HA cleavability. When the HA contained the carbohydrate, at least six basic amino acids had to be present at the cleavage site; however, when the carbohydrate was missing, they could be as few as four basic amino acids (17).

Current human influenza A viruses are not as virulent as the 1918 strain that killed 20 million people worldwide. The HAs of the human influenza A viruses are similar to those of avirulent avian strains in that the HA is not cleaved in tissue culture in the absence of trypsin (43). The HAs of human influenza A viruses do not contain a series of basic amino acids at the cleavage site but contain a single arginine and a carbohydrate in the near vicinity. The aim of this study was to examine the effects of introduction of a series of basic amino acids at the cleavage site and deletion of the carbohydrate on the susceptibility of the human influenza virus HA to cellular cleavage enzymes. Such information is important because it may indicate the potential of the virus to cause systemic infection in humans and thus may explain a sudden increase in virulence of a human influenza virus that was responsible for the 1918 pandemic.

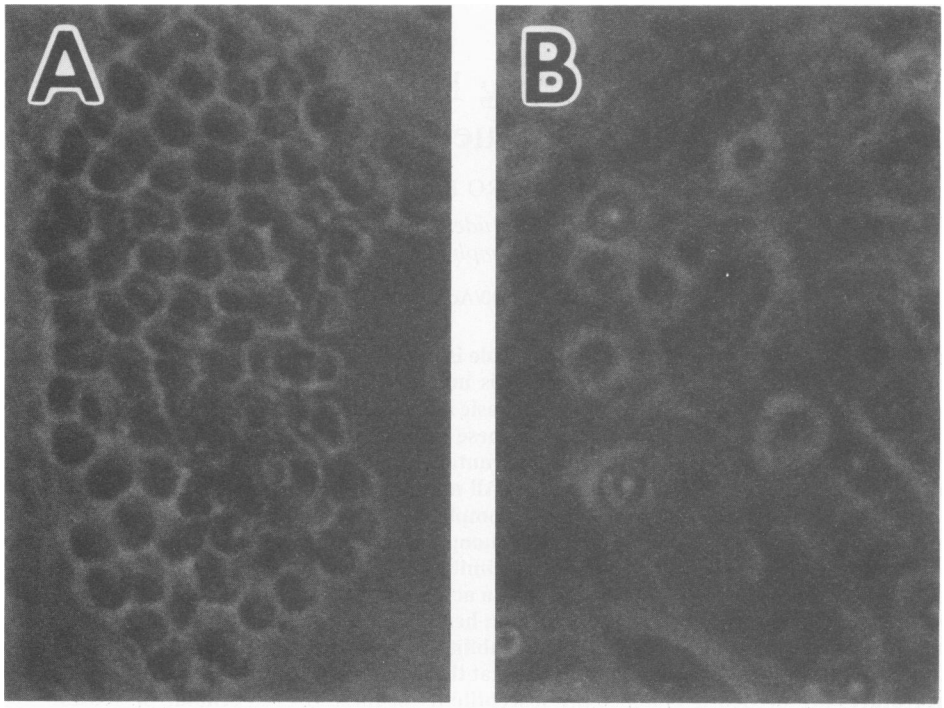

FIG. 1. Hemadsorption by the HA expressed in CV-1 cells infected with SV40-HA recombinant virus (A) or mock infected (B). At 72 h after infection with SV40-Aichi HA recombinant virus, cells were overlaid at room temperature with a 1% human erythrocyte suspension in isotonic phosphate-buffered saline; 20 min later, the unbound erythrocytes were removed by a wash with phosphate-buffered saline.

MATERIALS AND METHODS

Virus. A human influenza A virus, A/Aichi/2/68 (H3N2), was grown in 11-day-old embryonated chicken eggs and purified by differential sedimentation through 25 to 70% sucrose gradients in a Beckman SW28 rotor. Virion RNA was isolated by treatment of purified virus with protease K and sodium dodecyl sulfate, followed by extraction with phenol-chloroform (1:1), as previously described (1).

Cloning of the HA gene. Full-length cDNA was prepared by reverse transcription of virion RNA according to previously described methods (12). A 12-base synthetic oligonucleotide primer complementary to the 3′ terminus of the negative-strand RNA was phosphorylated with T4 polynucleotide kinase and then used to prime reverse transcription of the total virion RNA in the presence of [^{32}P]dATP. Second-strand DNA synthesis was performed with a phosphorylated 13-base synthetic primer complementary to the 3′ end of the cDNA and the Klenow fragment of *Escherichia coli* DNA polymerase I. Full-length double-stranded copies of the HA gene were blunt-end ligated into the *Pvu*II site of plasmid pATX, obtained from C. Naeve (St. Jude Children's Research Hospital, Memphis, Tenn.).

Construction of SV40-HA recombinant virus. The construction of simian virus 40 (SV40)-HA recombinant virus has been reported elsewhere (16).

Transfection and virus stocks. Seventy nanograms of recircularized SV40-HA recombinant DNA and the equivalent amount of DNA from the SV40 early deletion mutant *dl*1055 (35) were introduced into CV-1 cells by using DEAE-dextran (26). After 5 days at 37°C, the cells and medium were frozen and thawed three times, and fresh monolayers of CV-1 cells were infected with 0.3 ml of the resulting lysate. This procedure was repeated twice to obtain a virus stock.

Site-specific mutagenesis. The entire HA gene of A/Aichi/2/68 was subcloned in the double-stranded replicative form of M13mp19 phage DNA. The procedures for oligonucleotide-directed mutagenesis have been described in detail by Zoller and Smith (46). The entire HA gene of each SV40-HA recombinant was sequenced by the chain termination procedure (39) to ensure that only the desired changes were present in the molecules.

Assays for functions of HA. Hemadsorption and polykaryon formation by CV-1 cells infected with SV40-HA recombinant viruses were assayed as described previously (16).

RESULTS

A series of basic amino acids at the cleavage site results in partial cleavage of the HA of a human influenza A virus in the absence of trypsin. To determine whether the structural features required for cleavage of avian influenza virus HAs would lead to cleavage of the human influenza virus HAs, I cloned the HA gene of a human influenza A virus, A/Aichi/2/68 (H3N2), in a plasmid and expressed it in CV-1 cells, using the SV40 virus system. The expressed HA was transported to the cell surface, where it bound erythrocytes (Fig. 1) and showed fusion activity upon treatment with trypsin (Fig. 2), as has been shown by others (43). Without addition of trypsin, the HA was not cleaved, and only HA0 was detected on the gel in the absence of trypsin (Fig. 3, lane P). In the presence of the protease, however, the HA was cleaved into HA1 and HA2 (data not shown), confirming previous observations (43).

To investigate whether a series of basic amino acids would confer high cleavability to the HA of a human influenza A virus, as it does to avian influenza virus HAs (16), I constructed a mutant (MA7) that contained two amino acid

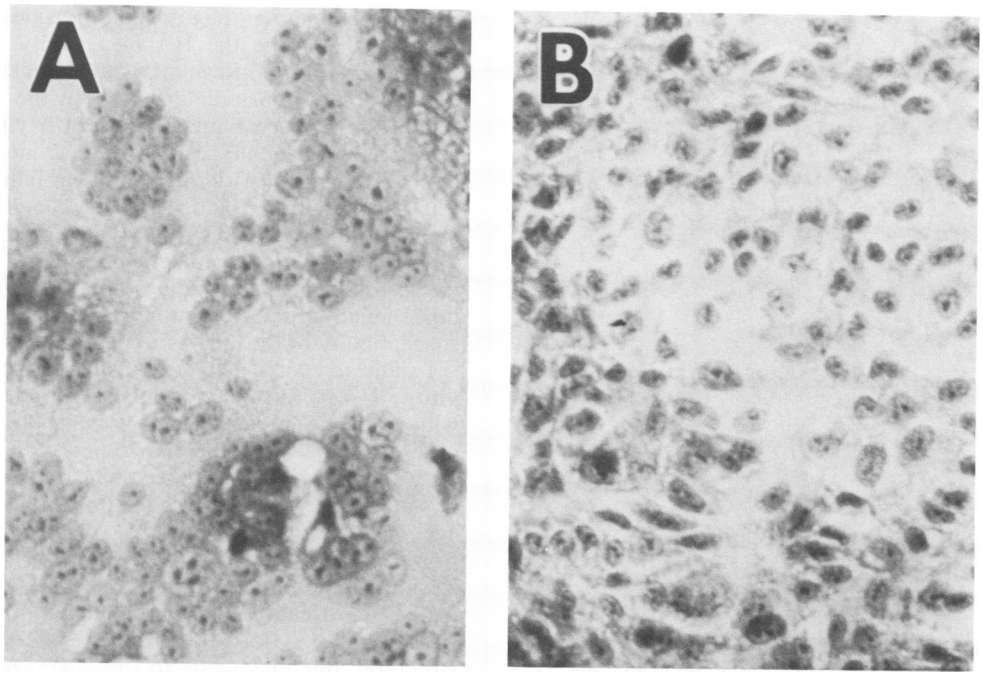

FIG. 2. Polykaryon formation by CV-1 cells infected with SV40-HA recombinant virus (A) or mock infected (B). At 72 h after infection with SV40-HA recombinant virus, the monolayers were treated with trypsin (2 μg/ml) for 20 min at 37°C and then incubated for 3 min at 37°C with phosphate-buffered saline (pH 4.8). Phosphate-buffered saline was then removed, and the cells were incubated for 7 h in tissue culture medium.

substitutions (nonbasic to basic) and Arg for Lys at the fourth residue from the carboxyl terminus of HA1 (Table 1), making this region identical to that of a virulent avian influenza virus, A/chicken/Scotland/59 (H5N1) (15). Another mutant (MA8) contained two additional basic amino acids besides those in MA7; the same sequence can be found at the cleavage site of a virulent avian influenza A virus, A/turkey/Ireland/1378/85 (H5N8) (15). The HAs of both virulent avian influenza viruses have been shown to be completely cleaved in the absence of trypsin or any other influenza virus genes (17). When produced in CV-1 cells in the absence of trypsin, only a portion (6%) of the MA7 HA was cleaved (Fig. 3). By contrast, 53% of the MA8 HA, which contained six basic amino acids, was cleaved. Both mutants showed all of the normal functions of the HA, including complete cleavage in the presence of trypsin (data not shown). When fusion activity was examined by polykaryon formation without trypsin treatment, approximately 20% of CV-1 cells expressing MA8 were fused, whereas no polykaryon formation was observed with the cells expressing the parent or MA7 HA (Fig. 4). This result indicates that a series of six basic amino acids at the cleavage site of a human influenza A virus HA will allow sufficient cleavage by cellular proteases to confer fusion activity.

An oligosaccharide at Asn-22 in the vicinity of the cleavage site does not interfere with HA cleavage of human influenza A virus A/Aichi/2/68. In the HAs of avian influenza viruses, the carbohydrate in the vicinity of the cleavage site interferes with HA cleavage when there are fewer than six basic amino acids at the cleavage site (17). To determine the importance of the carbohydrate for cleavage of human influenza virus HAs, I abolished the potential glycosylation site, Asn-22–Gly-23–Thr-24, by changing Thr-24 to Lys. The resulting mutant HA, MA9 (Table 1), which had lost the potential glycosylation site but contained the same amino acid sequence at the cleavage site as the parent, was not cleaved (Fig. 3) and did not show fusion activity (Fig. 4) in the absence of trypsin; however, the HA showed normal functions, including cleavage into HA1 and HA2 in the presence of exogenous trypsin. The mutant HA0 migrated faster than the parent HA0 (Fig. 3), suggesting that the carbohydrate was present at Asn-22 in the parent HA but not in the MA9 HA. These findings indicate that loss of the carbohydrate at Asn-22 does not by itself confer high cleavability to the HA of A/Aichi/2/68.

I then tested whether the presence of a series of basic amino acids at the cleavage site, together with loss of the carbohydrate, would confer high cleavability to the HA of a human influenza A virus. An amino acid substitution at position 24 from Thr to Lys was introduced into the two mutant HAs (MA7 and MA8) to abolish the potential glycosylation site (Table 1). Although the HAs of both of the resulting mutants (MA79 and MA89) had normal functions and were cleaved in the presence of trypsin (data not shown), they were still only partially cleaved in the absence of trypsin. An increase in the quantity of the cleaved product was not observed in the absence of the carbohydrate at Asn-22, compared with MA7 and MA8, which retained the potential glycosylation site (Fig. 3). The degree of polykaryon formation seen with MA89 was similar to that obtained with MA8, whereas with MA79 it was not detected (Fig. 4). Loss of the carbohydrate at Asn-22 was suggested by greater mobility of the HA1 molecules of MA79 and MA89 than those of MA7 and MA8. These results indicate that, in contrast to avian virus HAs, the carbohydrate at

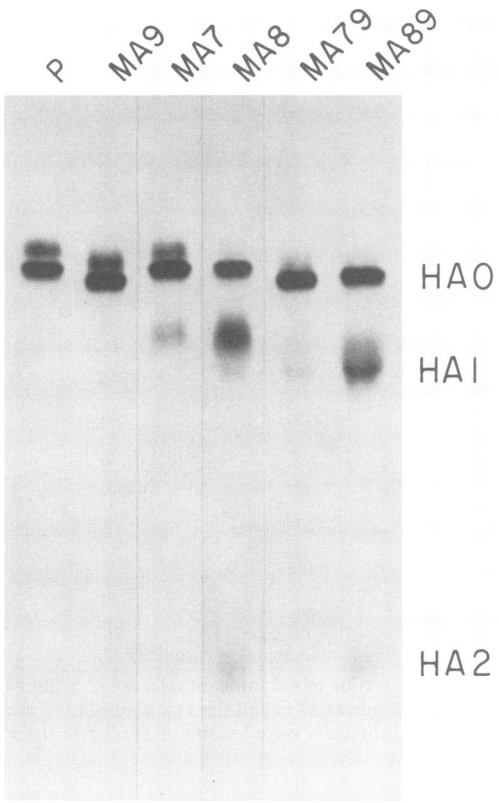

FIG. 3. Comparison of the HAs produced in SV40-HA. Cleavage of the HA molecule expressed in CV-1 cells was examined by infecting cell monolayers with SV40-HA recombinant virus; 36 h later, the HAs were labeled with [^{3}H]mannose and [^{3}H]glucosamine for 15 h at 37°C. Cell extracts were immunoprecipitated with monoclonal antibodies to the HA and analyzed on a 12.5% polyacrylamide gel as described previously (14). P, Parent.

Asn-22 does not interfere with cleavage of the A/Aichi/2/68 HA.

DISCUSSION

In previous studies (16, 17), we established that two structural features, a series of basic amino acids at the cleavage site and an oligosaccharide side chain in the near vicinity, determine the HA cleavability of virulent avian influenza A viruses. Results described in this report indicate that a series of basic amino acids at the cleavage site can render the HA of a human influenza A virus (A/Aichi/2/68) susceptible to cellular cleavage enzymes, although to a lesser extent than with virulent avian influenza virus HAs. The oligosaccharide side chain near the cleavage site, however, did not interfere with cleavage of the human influenza virus HA that contains a series of basic amino acids at the cleavage site. These findings indicate that other, as yet unidentified structural features are needed to enhance the susceptibility of these HAs to cellular proteases.

Failure to confer high cleavability to the human influenza viruws HA by introduction of structural features associated with virulence among avian viruses may reside in amino acid differences between the two types of HAs. Because the cleavage site is located in the middle of the stalk region of the HA molecule, the structural features required for HA cleavage most likely occur in that portion of the molecule. Comparison of amino acid residues in the stalk region (residues 11 to 51 [HA1] and 1 to 221 [HA2]) of A/Aichi/2/68 and a representative avian influenza A virus (A/turkey/Ireland/1378/85 [H5N8]) indicated a 54% difference. Hence, structural differences due to this divergence could explain why the oligosaccharide side chain did not interfere with cleavage of the human influenza virus HA and why the HA of A/Aichi/2/68 (H3N2) did not acquire cleavability similar to that of virulent avian influenza viruses, even though it met the same general structure requirements for HA cleavage as defined for the avian viruses.

Only the mutant HAs with six basic amino acids at the cleavage site showed limited fusion activity as assayed by polykaryon formation. It is not certain, however, whether this assay measures fusion activity representative of the HA on the virion. Possibly, the extent of cleavage required for the virus to be infectious differs from that required for the HA expressed on the cell surface to exhibit polykaryon formation. Indeed, the HA of a virulent avian influenza virus, A/turkey/Ontario/7732/66 (H5N9), is only partially cleaved, but the virus undergoes multiple replication without exogenous trypsin in tissue culture (15). Thus, it is not known how many HA molecules have to be cleaved to promote such an activity, either on the virion or on the cell surface. Whether the extent of cleavage observed with our mutant HAs is sufficient to make the virus biologically active

TABLE 1. Importance of basic amino acids at the cleavage site and the carbohydrate at Asn-22 for cleavage of A/Aichi/2/68 (H3N2) HA

Site-specific mutant	Amino acid sequence at:				Cleavage without trypsin[a]	Polykaryon formation without trypsin[b]
	Glycosylation site			Cleavage site[c]		
	22	23	24			
Parent	Asn	Gly	Thr	Glu------**Lys** Gln Thr **Arg**/Gly	−	−
MA7	Asn	Gly	Thr	Glu------[**Arg**] [**Lys**] [**Lys**] **Arg**/Gly	±	−
MA8	Asn	Gly	Thr	Glu [**Arg**] [**Lys**] [**Arg**] [**Lys**] [**Lys**] **Arg**/Gly	±	+
MA9	Asn	Gly	[Lys]	Glu------**Lys** Gln Thr **Arg**/Gly	−	−
MA79	Asn	Gly	[Lys]	Glu------[**Arg**] [**Lys**] [**Lys**] **Arg**/Gly	±	−
MA89	Asn	Gly	[Lys]	Glu [**Arg**] [**Lys**] [**Arg**] [**Lys**] [**Lys**] **Arg**/Gly	±	+

[a] −, Uncleaved; ±, partially cleaved.
[b] +, Approximately 15 to 20% of cells were fused; −, no polykaryon formation was observed.
[c] Dashes are included to adjust the sequence alignment; boldface letters indicate basic amino acids; slashes indicate the HA1/HA2 cleavage site. Mutated amino acids are boxed.

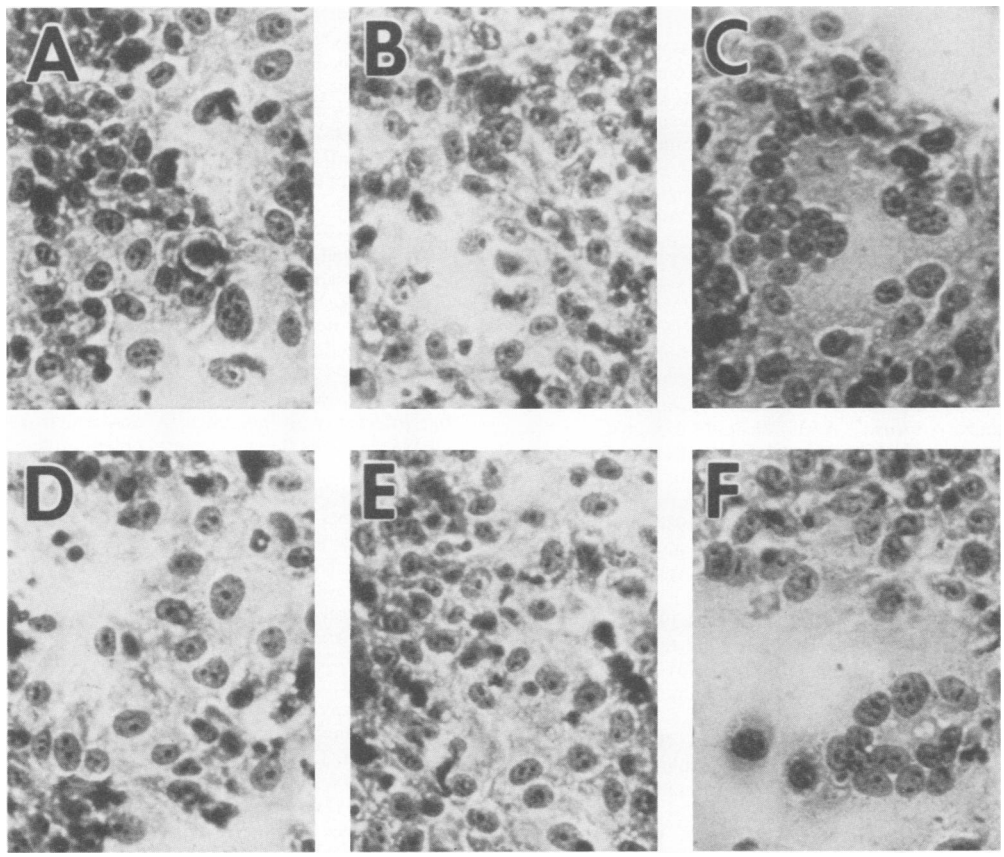

FIG. 4. Comparison of polykaryon formation by CV-1 cells infected with SV40-HA recombinant virus. At 72 h after infection with SV40-HA recombinant virus, the monolayers were incubated for 3 min at 37°C with phosphate-buffered saline (pH 4.8) without trypsin treatment. Phosphate-buffered saline was then removed, and the cells were incubated for 7 h in tissue culture medium. (A) Parent; (B) MA7; (C) MA8; (D) MA9; (E) MA79; (F) MA89.

cannot be determined in this system. The biologic significance of partial cleavage in our mutant HAs could be examined by construction of genetically engineered viruses, as recently described (7). However, serious consideration of the biohazard of such a virus might preclude its preparation.

At least two classes of influenza virus cleavage enzymes with different specificities are thought to exist in vivo. One cleaves the HA at a series of basic amino acids, while the other appears to have a relatively broad specificity that could encompass a series of basic amino acids as well as a single basic residue (3). The enzyme responsible for cleaving the HA with a series of basic amino acids appears specific for those HAs, based on findings in tissue culture (3). Many viral glycoproteins contain a series of basic amino acids at their cleavage sites (4, 9, 10, 32, 36, 42). Site-specific mutagenesis established that the presence of these amino acids is critical for cleavage (8, 11, 16, 25, 33, 34), suggesting the existence of multiple cleavage enzymes with similar specificities. Although enzymes that recognize multiple basic amino acids have been isolated (5, 6, 24, 41), they differ in their optimal pH and ion requirements, and it is not known whether they cleave influenza virus HAs or other viral glycoproteins. Rott et al. (37) isolated variants of a human influenza A virus, A/Aichi/2/68 (H3N2), whose HAs are cleaved in MDCK cells in the absence of trypsin. These mutants did not contain a series of basic amino acids at the cleavage site of the HA molecules; rather, a critical mutation seems to have occurred at amino acid residue 17 from histidine to arginine, which is located in the vicinity of the cleavage site in the three-dimensional structure. The HAs of these mutants, however, are not cleaved in other cell lines or in primary chicken embryo fibroblasts in the absence of trypsin. Similar findings were obtained with different influenza A viruses. Thus, the enzymes responsible for HA cleavage in vivo may differ according to the tissue source.

Results of pathologic studies of human tissue from the 1918 influenza pandemic suggested that the causative virus was neurotropic (13, 28), a characteristic associated with high cleavability of the HA (2, 27, 40). As demonstrated for the HAs of avian influenza viruses (16, 20), the capacity for high cleavability can reside in the HA molecule itself. Alternatively, the product of the influenza virus neuraminidase gene may be either directly or indirectly responsible for HA cleavage in a variety of cells (27, 40). Acquisition of virulence by alterations in HA cleavability has been shown to occur in avian influenza A viruses by either the loss of the carbohydrate in the vicinity of the cleavage site (14) or acquisition of multiple basic amino acids at that site (18, 23, 30, 31). Thus, a sudden increase in virulence of human influenza viruses, as illustrated by the 1918 pandemic strain, could result from acquisition of high cleavability by the HA molecule.

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