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REPLICATION OF INFLUENZA B VIRUS: BIOLOGICAL FUNCTIONS OF VIRAL NEURAMINIDASE

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INTRODUCTION

My virus research started at the Department of Bacteriology, Nagoya University School of Medicine as a postgraduate student in 1959. Initially, I took part in a series of Dr. Matsumoto's studies on host-controlled variation of paramyxovirus.[1] These studies provided me with the background necessary for virus research. After this work, I began to study persistent infection of cell culture by paramyxovirus similar in appearance to cell transformation by tumor viruses, under the leadership of Dr. Matsumoto[2] and continued the study at the Department of Virology (Prof. Matsumoto), Cancer Research Institute after I had finished the doctoral course. In 1966 I had the opportunity to study the replication of influenza virus at the Virus Division, Department of Public Health, Cornell University Medical College. Since then, I have contributed to the advance in knowledge of the molecular mechanism of influenza virus replication,[3–11] including the biological functions of viral proteins, virus morphogenesis, and formation of the nucleocapsid and its intracellular transport, while also studying the structure and assembly of paramyxoviruses[12–15] in parallel. Recent studies[4,11] of the function of influenza B virus neuraminidase using temperature-sensitive mutants, which Dr. Goshima in my laboratory took the major part in, have provided definite evidence that neuraminidase (NA) is closely related to virus morphogenesis, virus release from host cells, and cleavage of viral HA by host cell protease. Together with these findings, this article outlines only the biological functions of influenza virus NA that we have found until now, because of constraint of space.

A brief explanation of the structure and assembly of the influenza virus may be helpful for an understanding of this article (see Ref. 16). Fig. 1 illustrates the shape of the influenza virus. The virus genome consists of eight separated RNA molecules (RNA segments) of different sizes, each of which encodes one or two different polypeptides. The viral envelope encloses eight nucleocapsids, inside each of which is one RNA segment and many protein (NP) molecules. Three different P polypeptides —PA, PB1, and PB2— responsible for viral RNA synthesis are associated with each nucleocapsid. Glycoproteins, hemagglutinin (HA) and NA spikes are located on the surface of the viral envelope and the ends of these glycoproteins are embedded in the lipid bilayer derived from the plasma membrane of the host cells. HA is responsible for the attachment of the virus particle to the neuraminic acid-containing receptor on the host cell surface during the initial stages of infection and for the hemagglutination by the virus. NA acts to

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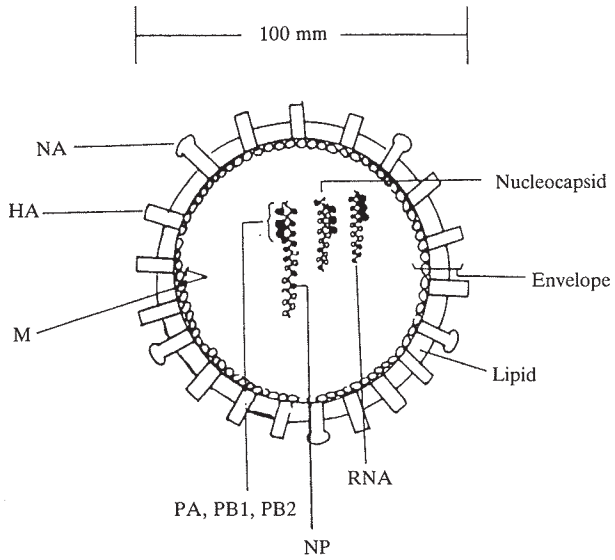

Fig. 1. Shape of influenza virus. The envelope encloses eight nucleocapsids inside, but only three nucleocapsids are shown in the figure.

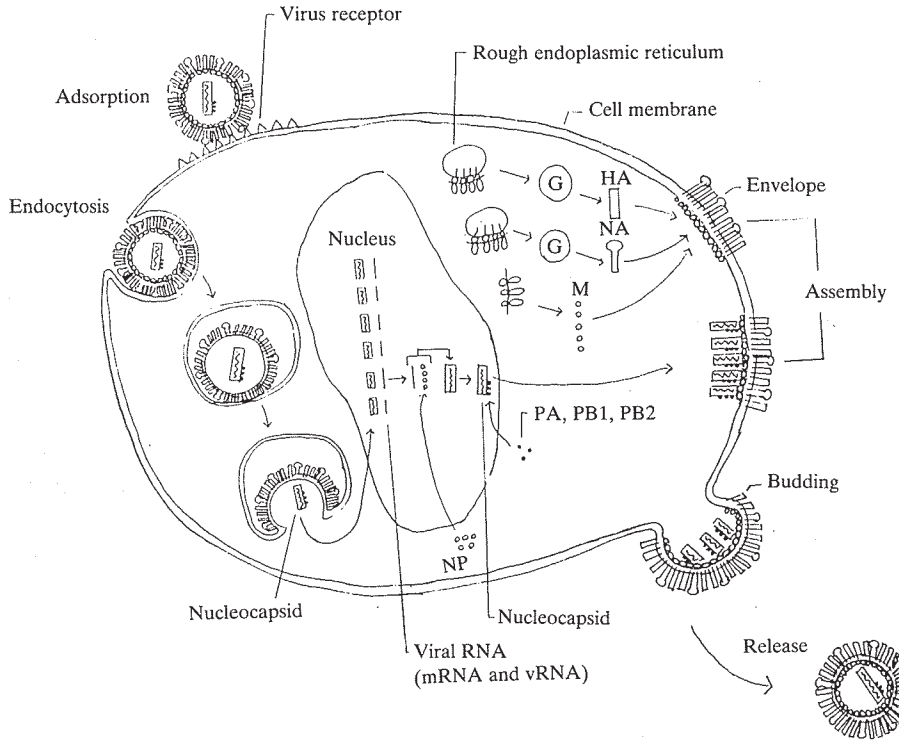

Fig. 2. Diagram of replication of influenza virus. Adsorption of input virus onto the receptor on host cell surface and the subsequent release of its nucleocapsids (genome) into the cytoplasm (uncoating) are shown at the left side of host cell, and the virus assembly and the subsequent release of progeny virus from host cell are shown at the right side.

liberate the terminal neuraminic acid residues (receptor for HA) from the carbohydrate moieties of glycoproteins. M protein associates with the inner surface of the lipid bilayer and determines the shape of the virus.

Influenza virus particles are formed on the plasma membrane of infected host cells (Fig. 2). The initial event in virus assembly is the insertion of HA and NA into the plasma membrane. HA and NA, after synthesis, are inserted into the membrane of the rough endoplasmic reticulum followed by transportation via the Golgi complex to the plasma membrane.[7] M protein, soon after synthesis, binds to the inner surface of the plasma membrane, resulting in the formation of a native viral envelope, just like the envelope of virus particles.[17] Eight RNA segments of the genome and their transcripts (messenger RNAs and template RNAs to the genome) are produced in the nucleus throughout infection. Genomic RNA segments, immediately after being synthesized, associate with newly synthesized NP molecules that are transported to the nucleus from the cytoplasm, resulting in the formation of eight nucleocapsids. Subsequently, three P proteins associate with each nucleocapsid and then the eight nucleocapsids are transported from the nucleus into the cytoplasm and bind to the M protein associated with the inner surface of the plasma membrane. Virus particles are formed on the plasma membrane by a budding process and are then released from the host cells.

[1] *The early event of infection does not depend on NA*

Sendai virus (Paramyxovirus family) exhibits hemolysis and fusion at neutral pH and can therefore transfer its genetic materials by fusion between the viral envelope and the plasma membrane of target cells, whereas other enveloped viruses including the influenza virus generally enter the cells by endocytosis, but by what mechanism the genomes of these viruses are released into the cytoplasm of host cells was unknown until 1980. At that time, Semliki Forest virus (SFV)[18] and subsequently vesicular stomatitis virus (VSV)[19] and influenza A viruses[20–23] were found to cause hemolysis and fusion by interacting with the cells in low pH environments (pH 5.0). These observations opened up a new aspect of the mechanism of infection by these viruses.

The studies with SFV[18] suggested that a fusion occurred between the viral envelope and the membrane of the intracellular endosomes into which the virus had been delivered, thereby releasing the nucleocapsid into the cytoplasm. The fusion is thought probably to be triggered by low pH in the endosomes, since lipophilic amines such as chloroquine, which raised the pH within the endosomes of macrophages, inhibit the release of the virus genome into the cytoplasm. Later, similar evidence[24,25] was obtained with influenza A virus (Fig. 2).

Proteolytic cleavage of HA of influenza A virus into disulfide-bonded HA1 and HA2 subunits is essential for its low pH-induced hemolysis and fusion activities[20–23] as well as the expression of infectivity,[26,27] because fusion activity of HA depends on a specific amino acid sequence at the amino terminus of HA2.[16] Extensive studies with influenza A virus by Haung et al. demonstrated that, in addition to cleaved HA, the enzymatic activity of NA was required for low pH-induced fusion by influenza A virus. Our previous experiment (unpublished data) showed that selective removal of NA from influenza B virus by trypsin treatment had no effect on its infectivity (the ability to produce progeny virus), suggesting that the virus may not require NA for its fusion with the membrane of endosomes. In addition, SFV and VSV, which differ from influenza viruses in the virus family, exhibit low pH-induced fusion, despite the lack of NA. These findings led us to confirm whether NA was actually required for low pH-induced fusion. For this, we examined low pH-induced hemolysis activity of NA-lacking influenza B virus, since the hemolysis by virus requires a single fusion event between the viral envelope and the cell membrane of the erythrocytes.[28]

(1) *Hemolysis and fusion activities of influenza virus depend only on HA activity.*[29]

Influenza B/Kanagawa/73 virus was grown in 10-day-old chick embryos to prepare cleaved HA spike-containing virus particles. The virus was allowed to adsorb onto chick erythrocytes at 4°C for 30 min. The erythrocytes were then washed and resuspended in saline buffered to pHs ranging from 5.0 to 7.2 for 2 min at 36°C and further incubated in phosphate-buffered saline (PBS, pH 7.2) at 36°C for 30 min. Hemoglobin released in the supernatant was determined from the A_{540} of the supernatant. The virus exhibited extensive hemolysis at pHs ranging from 5.0 to 5.9. The hemolysis induced by these pHs did not occur with erythrocytes in the absence of the virus nor with erythrocytes adsorbing the virus with uncleaved HA spikes, indicating that a low pH-induced hemolysis by influenza virus requires the cleavage of HA into HA1 and HA2.

When virus suspension was kept at 55°C, the virus lost more than 97% of its original enzymatic activity of NA within 10 min without any effect on hemagglutinating activity (HA activity). However, the heated virus suspension exhibited higher hemolytic activity at low pHs ranging from 5.0 to 5.9 than the unheated control.

Virus suspensions were treated with trypsin (20 to 250 μg/ml) at 36°C for 30 min to remove NA from the virus particles, followed by the addition of soybean trypsin inhibitor. The virus particles were sedimented by centrifugation at 25000 rpm for 1 h. The pellets were resuspended in the original volume of PBS and, together with the supernatants, tested for hemagglutinating and NA enzymatic activities. More than 99% of NA enzymatic activity was released from the virus particles and recovered in the supernantants, but hemagglutinating activity was recovered only in the sediment. This elimination of NA from virus particles had no effect on low pH-induced hemolytic activity but caused somewhat higher hemolysis. The treated virus did not cause hemolysis at pHs 6.0 and 7.2. The above findings indicate that cleaved HA, but not NA enzymatic activity, is essential for low pH-induced hemolysis and fusion by influenza B virus. Later, this was confirmed by White et al.,[30] who showed that cleaved HA expressed at the surface of the cells transformed by cDNA of the HA gene of influenza A virus induced extensive cell fusion at low pH.

(2) *Release of nucleocapsids of infecting virus into the cytoplasm*[10]

It had been reported that the growth of influenza A virus was inhibited in the presence of amantadine, but this was not the case with influenza B virus, suggesting that since the drug is a lipophilic amine, influenza B virus may differ from influenza A virus in the mechanism of release of nucleocapsids into the cytoplasm. We examined the effect of another lipophilic amine, chloroquine, on the growth of influenza B/Kanagawa/73 virus.

The addition of Chloroquine (100 μg/ml) to MDCK cell cultures before infection completely inhibited the virus yield, but this inhibitory effect was decreased when the drug was added later than 15 min after infection, indicating that chloroquine acts at a very early stage of virus infection. However, this inhibitory effect was reversible. The removal of the drug from the culture restored virus production with a 2-h delay. Electron microscopy showed that the appearance of infecting virus in the intracellular vesicles was not affected in the presence of the drug. RNA was extracted from infected cells and hybridized with ^{125}I-labeled vRNA from virus particles, and the RNase-resistant radioactivity was measured. Labeled vRNA was clearly protected from RNase by hybridization with RNA (cellular RNA plus viral RNA) from untreated, infected cells but not with the same amount of RNA from drug-treated, infected cells, indicating that the synthesis of RNAs complementary to genomic RNAs (mRNA and template RNA for synthesis of genomic RNAs) is inhibited in the presence of chloroquine.

Influenza B virus-infected cells were incubated in MEM containing cycloheximide (an inhibitor of protein synthesis) to limit the primary transcription (mRNA synthesis) of input virus

genome by nucleocapsid-associated RNA polymerase (PA, PB1, and PB2). At 4 h after infection, the medium was removed and the cells were pulse-labeled for 15 min with [^{35}S]methionine, followed by 10% SDS-PAGE. Viral polypeptides (HA, NP, NS, and M) were detected in significant amounts. In contrast, when chloroquine was added at the time of infection, the viral polypeptides were not detected, suggesting that either primary transciption or the translation of primary transcripts may be inhibited. However, these possibilities were unlikely. When the drug was added 30 min after infection, at which time viral polypeptides were undetectable, and the cells were labeled for 15 min with (^{35}S)methionine 4 h after infection, viral polypeptides were detected on gel. Altogether, the above results suggest that chloroquine inhibits infection by acting at a step preceding the primary transcription of the influenza B virus genome.

A pH-sensitive fluorescent probe was employed to measure the endosome pH of a suspension of living MDCK cells held in PBS and the pH change was quantitatively determined by fluorescence spectroscopy.[30] Chloroquine caused a rapid rise in the pH inside the endosomes of MDCK cells, to 6.5 from the physiological pH 5.6. This endosome pH dropped back to a value close to its initial value immediately after the removal of the drug from the medium. Since influenza B/Kanagawa virus exhibits fusion at pH 5.6 but not at pH 6.5,[29] it is most likely that influenza B virus releases its nucleocapsids into the cytoplasm by low pH-induced fusion between the viral envelope and the endosome membrane (Fig. 2) and that this fusion reaction is inhibited in the presence of chloroquine, due to the high pH environment inside the endosomes. Amantadine may have an inhibitory action distinct from other lipophilic amines.

[2] *Isolation and classification of temperature-sensitive mutants of influenza B/Kanagawa/73* (Goshima et al., submitted)

One approach to analyzing the biological function of viral proteins is to use temperature-sensitive (ts) mutants. Ts mutants of influenza A viruses have provided much valuable information on the molecular events involved in virus replication including the intracellular functions of viral proteins and cooperative interaction between viral proteins.[32] The virtue and versatility of ts mutants shown in influenza A viruses led us to isolate ts mutants of influenza B virus to facilitate our long-term study on its replication process.

Twenty-five ts mutants of influenza B/Kanagawa/73 generated by mutagenesis with 5-fluorouracil have been isolated. Wild-type virus produced highly infectious virus (10^6 to 10^7 PFU/ml) in MDCK cells at both 32°C and 37.5°C, while all mutants grew as well at 32°C as wild-type virus, but the yields of all mutants at 37.5°C were 10^{-3} or less than those at 32°C (see the yields of ts5 and ts7 in Tables 1, 3, and 4).

The influenza virus genome consists of eight separated RNA segments, each of which codes for one or more different polypeptides. Thus, pairwise crosses between two mutants result in the yields of recombinants of mixed genotype at high freqency, because influenza virus genetic recombination is essentially a random process. We could expect that when cells are mixedly infected with two ts mutants with ts lesion in the same RNA segment, non-ts recombinants are not produced at all. In contrast, pairwise crosses between two mutants with ts mutation in a different RNA segment result in the yield of non-ts recombinants at high efficiency. Thus, single ts mutants that have a ts lesion in only one RNA segment of the genome could be classified into eight groups, if the collection of ts mutants were enough to cover all RNA segments. However, our ts mutants were classified into seven groups on the basis of the presence or absence of non-ts recombinants in virus yields obtained by pairwise crosses between two ts mutants (Tables 1 and 2). Among 25 ts mutants, three mutants (ts5, ts7, and ts22) were single mutants that belonged to the same group and to none of the six other groups, and lacked NA enzymatic activity at the nonpermissive temperature.

Table 1. Recombination by Pairwise Crosses of Five Mutants*

Mutants**	16-h yield (PFU/ml) assayed at		% non-ts recombinants
	32°C	37.5°C	
14	1.3×10^7	$< 10^3$	
17	2.0×10^6	$< 10^3$	
7	6.6×10^6	$< 10^3$	
5	1.3×10^7	4.5×10^3	
8	9.6×10^6	$< 10^3$	
14 × 17	9.5×10^6	1.4×10^6	15
7 × 14	1.1×10^7	9.0×10^5	8.2
7 × 5	1.3×10^7	5.0×10^2	0
5 × 17	1.4×10^7	2.0×10^6	14
7 × 8	4.7×10^6	4.4×10^5	9.3

* MDCK cells were infected mixedly with two ts mutants and incubated at 32°C for 16 h. Infectivity titers (PFU) were assayed by plaque formation at 32°C and 37.5°C.

** ts7 and ts5 have a ts lesion in the same RNA segment and each of the other mutants has a ts lesion in a different RNA segment (Table 2).

Table 2. Classification of ts Mutants

Recombination groups	Ts mutants
I	1, 2, 10
II	11, 12, 18–409
III	14, 16, 9
IV	5, 7, 22
V	6, 8, 19, 18–407
VI	17
VII	18–438
I + II	4
III + V	20
IV + V	15
II + V + VII	18
III + V + VI	13

Table 3. Effect of Bacterial NA and Trypsin on Biological Activities of ts5 Produced at 32°C and 37.5°C

Temp. (°C)	Prepn examined	14—h virus yields*					
		No treatment		Bacterial NA		Bacterial NA + trypsin	
		HA	PFU/ml	HA	PFU/ml	HA	PFU/ml
32	fluid	64	3.8×10^7	64	7.1×10^7	64	3.0×10^7
	cells	64	1.1×10^7	64	2.4×10^7	64	1.5×10^7
37.5	fluid	<2	5.0×10^3	8	6.7×10^3	8	8.0×10^5
	cells	<2	4.3×10^4	16	5.0×10^4	16	9.4×10^6

* Fluids and cell lysates were untreated or treated with C. perfringens NA or C. perfringens NA plus trypsin and assayed for hemagglutinating activity and infectivity (PFU/ml).

Table 4. Effect of Bacterial NA and Trypsin on Biological Activities of ts7 Produced at 32°C and 37.5°C

Virus	Temp. (°C)	Prepn examined	Virus yields after 16 h						
			No treatment			Bacterial NA		Bacterial NA + trypsin	
			HA	NA*	PFU/ml	HA	PFU/ml	HA	PFU/ml
ts7	32	Fluids	64	0.000	3.1×10^6	64	9.9×10^6	64	1.0×10^7
		Cells	256	0.030	1.2×10^6	256	5.3×10^6	256	5.0×10^6
	37.5	Fluids	<2	0.000	$<10^3$	<2	$<10^3$	<2	$<10^3$
		Cells	<2	0.000	3.1×10^3	32	9.3×10^3	32	7.0×10^3
ts^+	32	Whole**	128	>1.0	1.4×10^7	128	2.0×10^7		
	37.5	Whole	128	>1.0	1.0×10^7	128	9.0×10^6		

* NA enzymatic activity was expressed at OD_{549} mu/0.1 ml.

** cell + culture fluid. ts^+: wild-type virus

[3] *NA serves for the late event of infection.*

It was found that the NA of influenza A virus facilitated the release of progeny virus from the host cell surface not only by the prevention of virus aggregation but also by the destruction of cellular neuraminic acid (receptor for virus).[33] The sixth RNA segments of influenza A and B viruses code for NA, but influenza B viruses differ from influenza A viruses in that the sixth RNA segments of influenza B viruses possess one additional gene coding for NB, which is not found in influenza A viruses.[34] NA and NB are translated from overlapping reading frames of a bicistronic mRNA transcribed from genomic RNA segment 6. NB, the third glycoprotein of influenza B viruses in addition to HA and NA, is an integral membrane protein expressed at the infected-cell surface and is not detected in virions.[35] Although it is likely that NB plays a role in the virus assembly process, its role in replication is not yet known. Influenza B virus NA may have a B-type virus-specific function not found in influenza A viruses, such as cooperation with NB. We characterized ts mutants ts5 and ts7 for analysis of roles of NA and NB in virus replication.

(1) *Characterization of ts5*[11]

When MDCK cells were infected with ts5 at 32°C, infectivity, hemagglutinating activity, and NA enzymatic activity appeared at 6 h and reached maximum levels at 12 h, whereas at 37.5°C these biological activities were negligible or considerably reduced (Table 3). However, neither protein synthesis nor transport of NA and HA to the cell surface were affected at 37.5°C (Fig. 2).

Ts5-infected cells were frozen and thawed 14 h after infection, and treated with bacterial NA, followed by treatment with trypsin (0.5 μg/ml). The biological activities of the cell lysate produced at 32°C were little affected by either treatment, while NA treatment of cell lysates obtained at 37.5°C restored hemagglutinating activity, and further treatment with trypsin resulted in the recovery of infectivity (Table 3). These results indicate that virus particles with uncleaved HA are fully produced at the nonpermissive temperature, since proteolytic cleavage of HA into HA_1 and HA_2 is required for expression of infectivity.

Scanning electron microscopy showed that a number of single virus particles were found on the surface of wild-type virus-infected cells at both temperatures (Fig. 3F) and ts5-infected cells at 32°C (Fig. 3C). In contrast, at 37.5°C, large aggregates of virus particles were found to accumulate on the surface of ts5-infected cells (Fig. 3D). The aggregates were broken up by the addition of bacterial NA to the culture for 30 min 9.5 h after infection, with concomitant appearance of hemagglutinating activity in the culture fluid (Table 3). Since HA binds to the terminal neuraminic acid residue (receptor for HA) on the oligosaccaride and NA acts to liberate the residue from oligosaccharide, one possible explanation for the above results is that (1) neuraminic acid-containing glycoproteins HA and NA of virus particles serve as the receptor for HA from other virus particles, resulting in aggregation; (2) virus particles in the virus aggregates are also bound to neuraminic acid on cellular glycoprotein or glycolipid; and (3) the lack of hemagglutinating activity is a consequence of formation of aggregates of progeny virus particles carrying neuraminic acid on their surface. Influenza B virus NA acts to prevent the aggregation of progeny virus particles by removing neuraminic acid from the viral envelope and cell receptor, resulting in release of the virus from the cells.

The proteolytic cleavage of HA spikes of virus particles is essential for expression of their infectivity,[26,27] and the cleavage of the HA by host cellular protease takes place at the infected cell surface.[27] The recovery of infectivity by trypsin treatment indicates that the cleavage of ts5 HA by host cellular protease is disturbed at the nonpermissive temperature. Since NA enzymatic activity of ts5 was absent under the nonpermissive condition, one possibility is that the terminal neuraminic acid residue on the oligosaccaride moiety of HA is responsible for interference with the subsequent cleavage of HA by host cellular protease. When bacterial NA was added to the culture infected with ts5 at 37.5°C 3 h after infection, the cells produced many infectious virus particles with hemagglutinating activity even at the nonpermissive temperature.

The open reading frames of the sixth RNA segments of wild-type virus and ts5 for translation begin at the first ATG codon (positions 47 to 49) and terminate at nucleotide 349 (Fig. 4A and B). These open reading frames encoding NB polypeptide of 100 amino acids have one nucleotide change between two viruses at position 253, but this change resulted in no amino acid change (Fig. 4B). The second ATG codon (positions 54 to 56) is followed by an open reading frame, extending to nucleotide 1454, which corresponds to the NA gene (Fig. 4A). Eight nucleotide changes between the two viruses were noted and five nucleotide changes resulted in amino acid substitutions. Four potential glycosylation sites (Asn-X-Ser/Thr) and 18 cysteine residues encoded by the wild-type virus were conserved in ts5.

At the nonpermissive temperature, ts5 produced noninfectious virus particles which became readily infectious after treatment with trypsin, suggesting that under nonpermissive conditions,

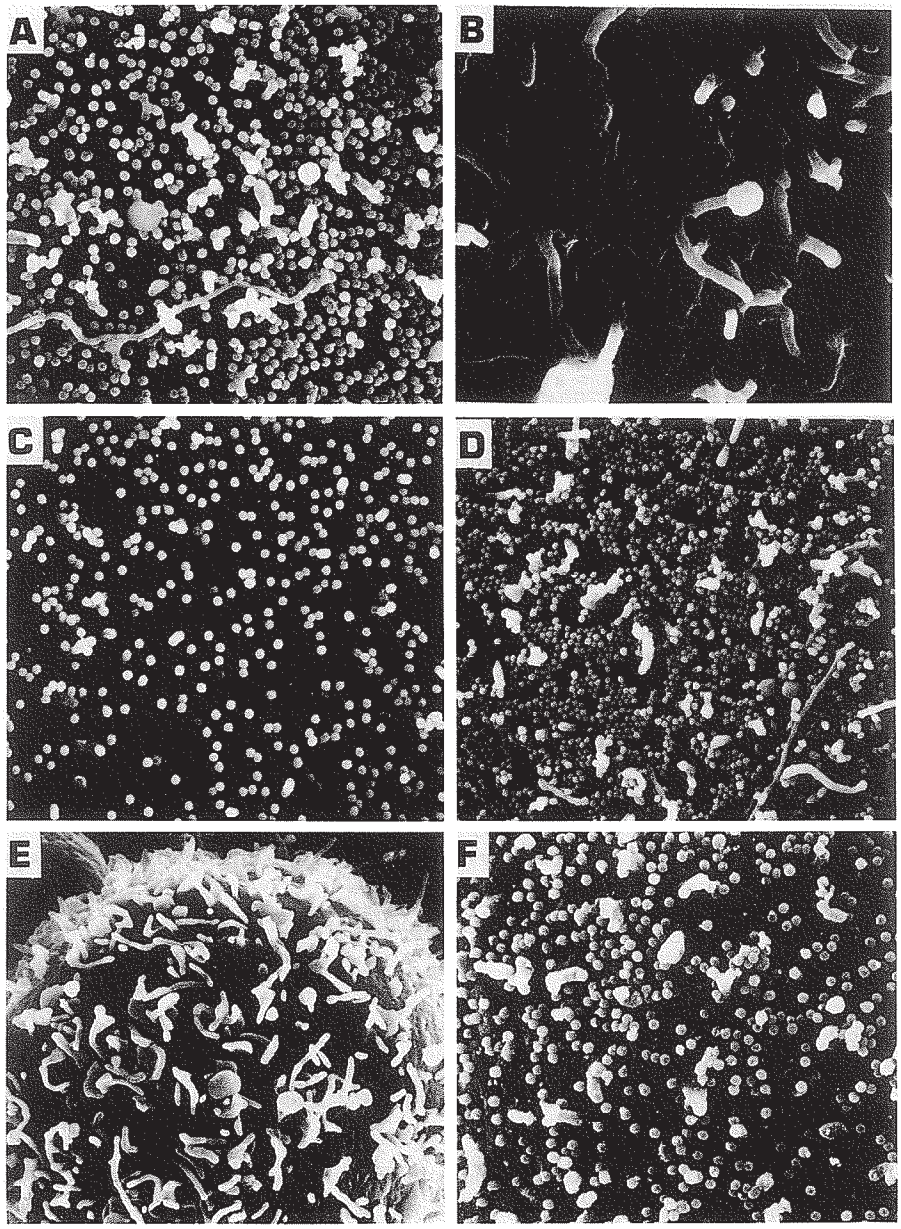

Fig. 3. Scanning electron microscopy. MDCK cells were infected with each wild-type virus, ts5 and ts7, and incubated at 32° and 37.5°C for 10 h. The cells were processed for scanning electron microscopy. Cells infected with ts7 at 32°C (A) and 37.5°C (B); cells infected with ts5 at 32°C (C) and 37.5°C (D); Cells infected with wild-type virus at 37.5°C (F); Uninfected cells (E). Magnification: (A, B, C, D, and F) ×20.000; (E) ×8.000.

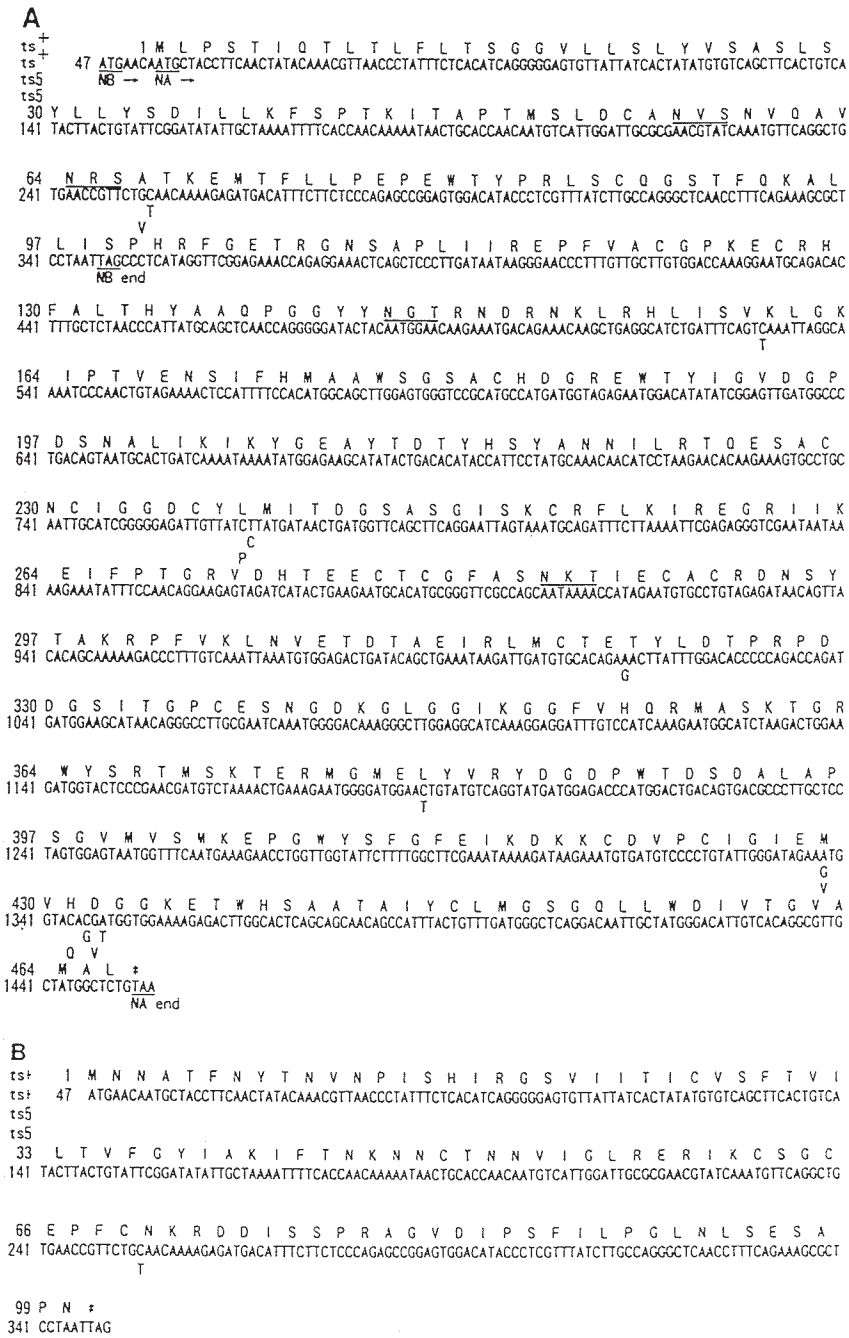

Fig. 4. (A) Nucleotide sequences of the coding regions of the sixth RNA segments and deduced amino acid sequences. The sequences of the coding regions of RNA segment 6 from wild-type virus and ts5 are shown in the mRNA sense, and numbering is from the first nucleotide of the noncoding region. The amino acid sequence of the NA polypeptide was deduced from the nucleotide sequence at positions 54−1454. The sequence of NB gene starts at nucleotide position 47 and terminates at position 349. The positions of potential glycosylation sites are underlined. (B) Nucleotide and deduced amino acid sequences of the NB genes of wild-type virus and ts5.

all viral proteins necessary for replication are produced and functional except for NA and HA. Since treatment with bacterial NA restored hemagglutinating activity, the lack of hemagglutinating activity is probably a secondary effect that results from the lack of NA enzymatic activity. Thus, it can be concluded that ts5 has a ts defect in the NA molecule.

(2) *Characterization of ts7*[5]

Ts7 was also defective in NA enzymtic activity and hemagglutinating activity at the nonpermissive temperature (Table 4), though the viral protein synthesis was not affected. However, ts7 differed from ts5 in that both transport of NA of ts7 to the cell surface and formation of its progeny virus on the cell surface were disturbed at the nonpermissive temperature (Fig. 3A and B). To determine whether ts7 has a ts lesion only in RNA segment 6, we examined the genome composition of recombinants possessing a non-ts phenotype obtained by cross between ts7 and influenza B/Lee/40 strain. Gel electrophoresis of genomic RNA segments of Lee and ts7 showed that any RNA segment of the Lee virus genome did not comigrate with the corresponding RNA segment of ts7, allowing us to determine the origin of each RNA segment of non-ts recombinants. All non-ts recombinants tested had RNA segment 6 from the B/Lee genome and three of them (8-3-1, 8-2-1B, 9-2-M) inherited all RNA segments from ts7, except for RNA segment 6 (Table 5), indicating that ts7 has a ts lesion only in RNA segment 6.

RNA segment 6 of influenza B virus has two genes coding for NA and NB. To determine whether ts7 also has a ts lesion in the NB gene, the nucleotide sequences of coding regions of the sixth RNA segments of wild-type virus and ts7 were analysed. No nucleotide change was found in the region of the NB gene between both viruses (Table 6). However, there were six nucleotide changes in the region of the NA gene that was not overlapped by the NB gene, and five nucleotide changes resulted in four amino acid changes at 237, 395, 458, and 463, indicating that ts7 has a ts defect only in the NA molecule. Therefore, it can be concluded that the insertion of NA into the plasma membrane is essential for formation of the virus particle. Several lines of evidence have demonstrated that influenza A viruses are formed on the plasma membrane of infected cells even when transport of NA to the cell surface is blocked.[36–38] Since influenza B virus expresses NB as well as NA at the plasma membrane, assembly or budding of influenza B virus on the plasma membrane might be required for the interaction between NA and NB. Ts7 NA would serve as an effective tool for the understanding of intracellular transport of cellular glycoproteins.

The NA spikes associated with virus particles are formed as a tetramer of four identical

Table 5. Genome Composition of ts^+ Recombinants Obtained by Crossing Between ts7 and Wild-type B/Lee/40

ts^+ Recombinants	Origin of RNA segments							
	1	2	3	4	5	6	7	8
R23	L*	L	L	K	K	L	K	K
9-2-3B	K	L	L	K	K	L	L	L
9-2-5	K	K	K	K	K	L	L	K
8-3-1	K	K	K	K	K	L	K	K
8-2-1B	K	K	K	K	K	L	K	K
9-2-1M	K	K	K	K	K	L	K	K

* K: RNA segment derived from ts7 mutant;
L: RNA segment derived from wild-type B/Lee strain.

Table 6. Mutation of Open Reading Frame of RNA Segment 6 of ts7

Region of gene	Nucleotide sequence		Amino acid sequence	
	Position	Substitution	Position	Substitution
NB start	47 – 49	ATG		
NA start	54 – 56	ATG	1	
NB end	347 – 349	TAG		
	762	TAT → CAT	237	Tyr → His
	1236	GCT → ACT	395	Ala → Thr
	1426	ATT → ACT	458	Ile → Thr
	1436	GGC → GGT	461	silent
	1441] 1442]	GCT → GAC	463	Ala → Asp
NA end	1452 – 1454	TAA		

subunits and have a mushroom-shaped structure with a stalk and a head. The active site of the enzyme is located at the surface of the head of each monomer.[39] The monomeric head of NA is made up of six β-sheets, each containing four antiparallel strands connected by loops of variable sizes.[39,40] Ts5 NA monomer has four amino acid changes at positions 238, 429, 431, and 432 within its head domain. These mutations are located at two β-sheets (β 3S2 for position 238 and β 6S2 for positions 429 and 431) and a loop (position 432) between strands β 6S2 and β 6S3 in the three-dimensional structure of NA of influenza virus strain B/Beijiing/1/87 in the nomenclature of Burmeister et al..[40] Two of four mutations of ts7 NA monomer are located at two β-sheets (β 3S2 for position 237 and β 5S4 for position 395) and the other two are located at a different loop. Thus, amino acid change at position 395 on β 5S4 was specific for ts7 NA monomer but not found in ts5 NA monomer. The structural integrity of the head domain is maintained, at least in part, by β-sheets, whereas more structural flexibility is allowed in the connecting loops. In both influenza A and B viruses, loop mutations are responsible for much of the antigenic variation between field strains as well as antigenic variation against monoclonal antibodies.[39] Mutations in the loop region may be nonessential for the ts phenotype. Therefore, amino acid change at position 395 in β 5S4 of ts7 NA monomer may result in a ts defect in transport of NA to the cell surface.

(3) *Neuraminic acid on carbohydrate moieties of HA interferes with its cleavage by host cell protease* (Goshima and Maeno, submitted)

We also examined whether the HA expressed at the ts7-infected cell surface required NA enzymatic activity for its cleavage by host cell protease. MDCK cells were infected with wild-type virus at 32°C and 37.5°C or with ts7 at 32°C for 10 h after infection and exposed to sodium acetate-buffered saline pH 5.2 for 2 min. Under these conditions, all cells fused with each other to form polykaryocytes (Fig. 5A), while the cells infected with ts7 at 37.5°C exhibited no low pH-induced cell fusion (Fig. 5B). However, the pretreatment with external trypsin caused extensive cell fusion (Fig. 5D), indicating that HA at the ts7-infected cell surface at 37.5°C is in an uncleaved form. When RNA segment 6 of ts7 was replaced with that of wild-type B/Lee, the recombinants (8-3-1, 8-2-1B, 9-2-1M in Table 5) produced functional NA and HA at 37.5°C and caused low pH-induced fusion at the same levels as the wild-type parent virus. Bacterial NA was added to the culture infected with ts7 at 37.5°C 3 h after infection and incubated for

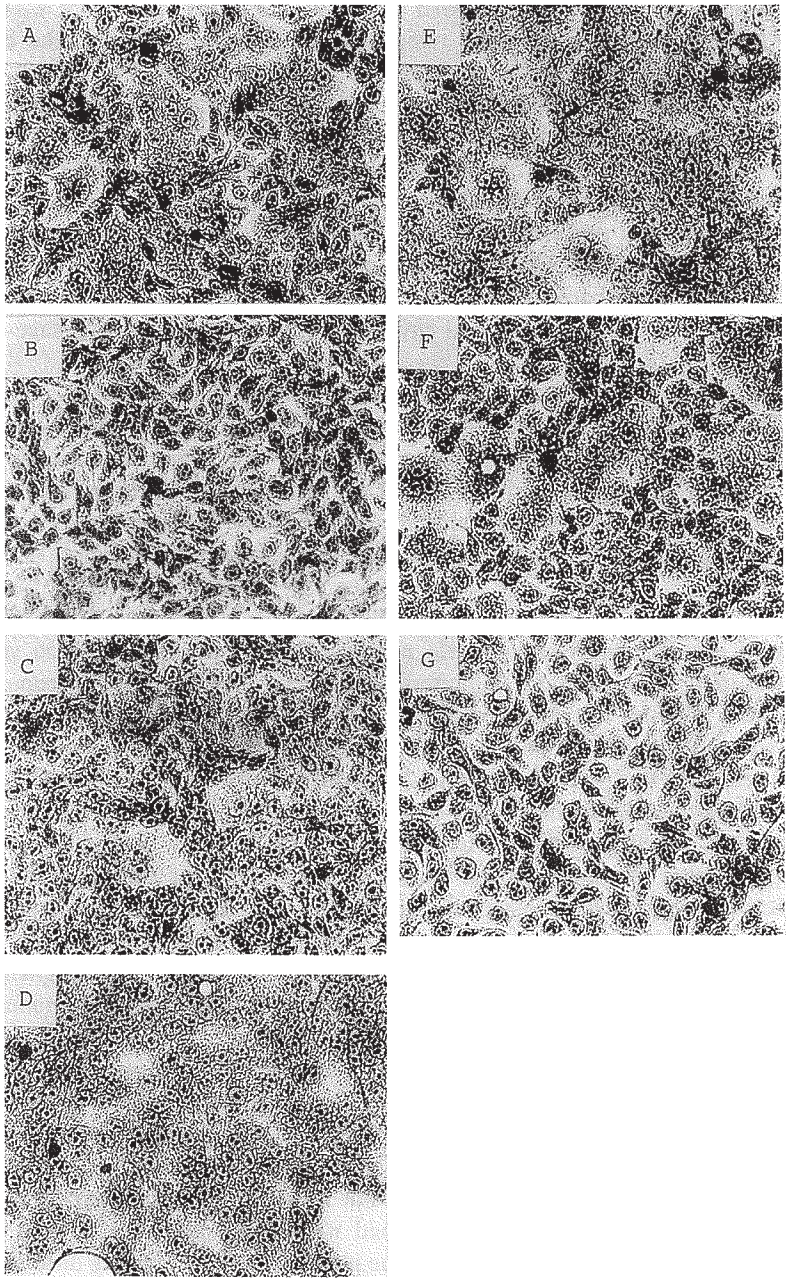

Fig. 5. Low pH-induced cell fusion of MDCK cells infected with either wild-type virus or ts7. MDCK cells were infected with either wild-type virus or ts7 at 5 PFU/ml and incubated at 32°C and 37.5°C for 10 h. C. perfringens NA was added to one portion of cultures infected with ts7 at 37.5°C from 3 h up to 10 h after infection (C) or trypsin was added for 30 min 10 h after infection (D). The cells were exposed 2 min to 0.01M sodium acetate-buffered saline (pH 5.2) and further incubated in MEM containing 5% FCS at 34°C for 3 h, followed by Giemsa staining. Cells infected with wild-type virus at 32°C (E) and 37.5°C (F) and with ts7 at 32°C (A) and at 37.5°C (B); uninfected cells (G).

another 7 h. These cells fused with each other after exposure to pH 5.2 (Fig. 5C). The above results supported our hypothesis that the removal of neuraminic acid from the carbohydrate moiety of HA by NA is essential for the subsequent cleavage of HA by host cell protease. In contrast, when bacterial NA was added for 30 min 16 h after infection, hemagglutinating activity of frozen and thawed cell lysate was recovered, but low pH-induced cell fusion did not occur. The cleavage of HA by host cell protease takes place at the plasma membrane[27], and when the native viral envelope is formed at the plasma membrane, host cell proteins are excluded from the membrane, which is a precursor to the viral envelope.[17] One possible explanation for the above result is that all of the HA molecules synthesized have already been inserted into the native viral envelope when bacterial NA is added late in infection, so that host cell protease is inaccessible to the HAs at the native viral envelope and thus proteolytic cleavage of HA does not occur.

In summary, the NA of influenza B virus is essential for the late event of infection but not for the early event. It was found that the NA of influenza A virus facilitated the release of progeny virus from the host cell surface not only by prevention of virus aggregation but also by the destruction of cellular neuraminic acid[33] (receptor for virus). Our studies have confirmed these findings and have presented the additional evidence that the NA of influenza B virus is essential not only for the cleavage of HA by host cell protease but also for virus assembly or budding.

[3] *Direct inactivation of influenza B virus by complement: Involvement of HA and NA*[41,42]

A variety of normal animal sera contain non-specific inhibitors of influenza viruses. They have been classified as α, β, and γ inhibitors, depending on their chemical composition and properties. The α inhibitor is a sialylated (neuraminic acid-containing) glycoprotein that inhibits hemagglutination by influenza A and B viruses but, unlike β and γ inhibitors, does not prevent infection. Its activity is destroyed by bacterial NA but not by heating for 30 min at 56°C. The γ inhibitor resembles the α inhibitor in most of its properties. In contrast, the β inhibitor is inactivated by heat (56°C for 30 min) but not by bacterial NA.[44]

It was reported that certain enveloped viruses interacted with the complement system, without the participation of antibody, to activate the classical complement pathway and/or the alternative complement pathway, leading to direct inactivation of the viruses.[45] Judging from physicochemical properties, it seemed likely that the β inhibitor was part of the complement system. This possibility was examined using normal guinea-pig sera (GPS), because all human sera must contain antibodies against influenza viruses by natural infection.

When influenza B/Kanagawa virus suspension (7.2×10^7 PFU/ml) was mixed with an equal volume of normal GPS and incubated at 36°C for 30 min, infectivity titer was reduced to more than 100-fold (5.5×10^4 PFU/ml) and hemagglutinating activity was also lost. This inactivation did not occur when the mixture was incubated at 4°C or when GPS had been heated at 56°C for 30 min, suggesting that neutralizing antibody is not responsible for the inactivating effect observed. Essentially the same results were obtained when other influenza B virus strains were used, and there was no significant difference in the inactivation effect among different batches of GPS. A 1:10 dilution of GPS was the saturating dose for its inactivation effect, but 1:100 diluted GPS showed no appreciable effect. The inhibitor was also sensitive to trypsin digestion. Interestingly, GPS did not inactivate influenza A virus.

GPS did not inactivate the virus in the presence of EDTA, but addition of $CaCl_2$ and $MgCl_2$ to EDTA-treated GPS restored its inactivation activity. When Mg^{2+} and EGTA were added to GPS instead of EDTA, the GPS also failed to inactivate the virus, but its activity was restored by the addition of $CaCl_2$. These results indicate that Ca^{2+} is required for inactivation by GPS. If complement was involved, the pathway of action would be the classical one, which is dependent

on both Ca^{2+} and Mg^{2+} ions, rather than the alternative pathway, which requires only Mg^{2+}.[45] The initial activation of the classical complement pathway converts complement component C1 to its activated state in the presence of Ca^{2+} and activated C1 then cleaves C4 and C2, the next two reacting components, to form C4b2a in a reaction depending on Mg^{2+}. Finally, the membrane attack pathway is initiated by C5b.[45] The serum obtained from guinea-pigs with heriditary complement component C4 deficiency showed no appreciable inactivation effect, but when C4 was added to the C4-deficient GPS, the reconstituted serum significantly reduced the infectivity titer.

A study was carried out to determine whether HA or NA on the viral envelope triggered the activation of the classical complement pathway. The enzyme-active portion of the NA molecule was selectively removed from influenza B virus by trypsin digestion. However, the virus was inactivated to a level comparable to untreated virus, suggesting that inactivation by GPS is induced by the interaction of HA with the inhibitor.

Complement component C1 alone bound directly to influenza B virus and inhibited its hemagglutinating activity. We suggest that the β-like inhibitor in GPS is a component of the classical complement pathway that is triggered by HA, leading to virus inactivation.

When influenza B virus was incubated with GPS at 0°C or 37°C for 30 min, hemagglutinating activity was completely inhibited. However, the addition of EDTA after incubation at 0°C restored hemagglutinating activity, but neither hemagglutinating activity nor infectivity was recovered by the addition of EDTA after incubation at 37°C. The first complement C1 inhibited hemagglutinating activity independently of incubation temperature, but hemagglutinating activity was readily recovered by the addition of EDTA at either incubation temperature. It is probable that the interaction of C1 with the virus sterically hindered binding of HA to the receptor on red cells. This seemed likely, because GPS C1q antigen was found associated with the virus treated with C1 by peroxidase-conjugated antibody technique. The inactivation of influenza B virus by GPS appeared to result from the steric hindrance of HA activity by association with serum proteins presumed to be complement components. However, trypsin digestion of the associated proteins fully restored the hemagglutinating activity but not the infectivity. The classical complement pathway is generally triggered by an antibody complexed with viral antigen and responsible for cleavage of complement component C5 essential for the initiation of the membrane attack pathway. If the cleavage of C5 occurs on the viral envelope and the resulting C5b immediately binds to the membrane, the protein assembles with late complement components C6-9 into a large multimolecular complex responsible for the lysis of the membrane, thereby inactivating the virus. It was found that GPS-treated virus underwent minor disruption of the envelope and degradation of M protein and genomic RNA.

The above results can be understood as indicating that the classical complement pathway activated by influenza B virus HA initiates the membrane attack pathway responsible for the envelope lesion and that this allows external proteolytic enzyme and RNAase to enter and degrade M protein and viral genome. Since the complement recognizes influenza B virus directly in the absence of antibody and inactivates it, the complement may play a role in primary host defence.

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REFERENCES

1) Matsumoto, T. and Maeno, K.: A host-induced modification of hemagglutinating virus of Japan (HVJ, Sendai virus) in its hemolytic and cytopathic activity. *Virology*, 17, 563–570 (1962).
2) Maeno, K., Yoshii, S., Nagata, I., and Matsumoto, T.: Growth of Newcastle disease virus in a HVJ carrier culture of HeLa cells. *Virology*, 29, 255–263 (1966).
3) Aoki, H., Nishiyama, Y., Tsurumi, T., Shibata, M., Ito, Y., Seo, H., Yoshii, S. and Maeno, K.: Mechanism of interference between influenza A/WSN and B/Kanagawa virus. *J. Gen. Virol.*, 65, 1385–1393 (1984).
4) Yamamoto-Goshima, F. and Maeno, K.: Persistent infection of MDCK cells by influenza C virus: Initiation and characterization. *J. Gen. Virol.*, 70, 3481–3485 (1989).
5) Yamamoto-Goshima, F., Maeno, K., Morishita, T., Ueda, M., Fujita, Y., Nakajima, K., and Yoshii, S.: Role of neuraminidase in the morphogensis of influenza B virus. *J. Virol.*, 68, 1250–1254 (1994).
6) Hamaguchi, M., Maeno, K., Yoshida, T., Nagai, Y., Iinuma, M. and Matsumoto, T.: Analysis of nuclear accumulation of influenza nucleoprotein antigen using a temperature-sensitive mutant. *Microbiol. Immunol.*, 29, 1131–1137 (1985).
7) Maeno, K. and Kilbourne, E.D.: Developmental sequences and intracellular sites of synthesis of three structual protein antigens of influenza A_2 virus. *J. Virol.*, 5, 153–164 (1970).
8) Maeno, K., Yoshii, S., Yoshida, T., Kawamoto, Y., and Matsumoto, T.: Intracellular development of membrane protein. *Microbiol. Immunol.*, 21, 427–438 (1977).
9) Maeno, K., Yoshii, S., Mita, K., Hamaguchi, M., Yoshida, T., Iinuma, M., Nagai, Y. and Matsumoto, T.: Analysis of the inhibitory effect of canavanine on the replication of influenza RI/5^+ virus. 1. Inhibition of assembly of RNP. *Virology*, 94, 128–137 (1979).
10) Shibata, M., Aoki, H., Tsurumi, T., Sugiura, Y., Nishiyama, Y., Suzuki, S. and Maeno, K.: Mechanism of uncoating of influenza B virus in MDCK cells: Action of chloroquine. *J. Gen. Virol.*, 64, 1149–1156 (1983).
11) Shibata, S., Goshima-Yamamoto, F., Maeno, K., Hanaichi, T., Fujita, Y., Nakajima, K., Imai, M., Komatsu, T. and Sugiura, S.: Characterization of a temperature-sensitive influenza B virus mutant defective in neuraminidase. *J. Virol.*, 67, 3264–3273 (1993).
12) Maeno, K., Yoshida, T., Iinuma, M., Nagai, Y., Matsumoto, T. and Asai, J.: Isolation of hemagglutinin and neuraminidase subunits of hemagglutinating virus of Japan. *J. Virol.*, 6, 492–499 (1970).
13) Iinuma, M., Yoshida, T., Nagai, Y., Maeno, K., Matsumoto, T., and Hoshino, M: Subunits of NDV. Hemagglutinin and neuraminidase subunits of Newcastle disease virus. *Virology*, 46, 663–677 (1971).
14) Iinuma, M., Maeno, K., and Matsumoto, T.: Studies on the assembly of Newcastle virus: An arginine-dependent step in virus replication. *Virology*, 51, 205–215 (1973).
15) Yoshida, T., Nagai, Y., Yoshii, S., Maeno, K. and Matsumoto, T.: Membrane (M) protein of HVJ (Sendai virus): Its role in virus assembly. *Virology*, 71, 143–161 (1976).
16) Kilbourne, E.D.: *Influenza* (1987). Plenum Medical Book Company, New York and London.
17) Hay, A.J.: Studies on the formation of the influenza virus envelope. *Virology*, 60, 398–419 (1974).
18) Helenius, A., Kartenbeck, J., Simons, K. and Fries, E.: On the entry of Semliki Forest virus into BHK-21 cells. *J. Cell Biol.*, 84, 404–420 (1980).
19) White, J., Matlin, K. and Helenius, A.: Cell fusion by Semliki Forest, influenza, and vesicular stomatitis viruses. *J. Cell Biol.*, 89, 674–679 (1981).
20) Huang, R.T.C., Wahn, H., Klenk, H.D. and Rott, R.: Fusion between cell membrane and liposomes containing the glycoproteins of influenza virus. *Virology*, 104, 294–302 (1980a).
21) Huang, R.T.C., Rott. R., Wahn, K., Klenk, H.D. and Kohama, K.: The function of the neuraminidase in membrane fusion induced by myxoviruses. *Virology*, 107, 313–319 (1980b).

22) Huang, R.T.C., Rott, R. and Klenk, H.D.: Influenza viruses cause hemolysis and fusion of cells. *Virology*, 110, 243–247 (1981).
23) Maeda, T. and Ohnishi, S.: Activation of influenza virus by acidic media causes hemolysis and fusion of erythrocytes. *FEBS Letters*, 122, 283–287 (1980).
24) Matlin, K.S., Reggio, H., Helenius, A. and Simons, K: Infectious entry pathway of influenza virus in a canine kidney cell line. *J. Cell Biol.*, 91, 601–613 (1981).
25) Yoshimura, A., Kuroda, K., Kawasaki, K., Yamashina, S., Maeda, T. and Ohnishi, S.: Infectious cell entry mechanism of influenza virus. *J. Virol.* 43, 284–293 (1982).
26) Klenk, H.D., Rott, R., Orlich, M. and Bloedorn, J.: Activation of influenza A viruses by trypsin treatment. *Virology*, 68, 426–439 (1975).
27) Lazarowitz, S.G. and Choppin, P.W.: Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. *Virology*, 68, 440–454 (1975).
28) Bachi, T., Deas, J.E. and Howe, C.: *Virus infections and the cell surface review*, Vol.2, pp.83–126 (1977). edited by Poste, G. and Nicholson, G.L., New York: North-Holland.
29) Shibata, M., Maeno, K., Tsurumi, T., Aoki, H., Nishiyama, Y., Ito, Y., Isomura, S. and Suzuki, S.: Role of viral glycoproteins in haemolysis by influenza B virus. *J. Gen. Virol.*, 59, 183–186 (1982).
30) White, J.M., Helenius, A.A., and Gething, M.J.: The haemagglutinin of influenza expressed from a cloned gene promotes membrane fusion. *Nature*, 300, 658–659 (1982).
31) Ohkuma, S. and Poole, B.: Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. U.S.A.*, 75, 3327–3331 (1978).
32) Mahy, B.W.J.: Mutants of influenza virus. In *Genetics of influenza viruses*, edited by Palese, P. and Kingsbury, D.W., pp.192–254 (1983). Springer-Verlag, New York.
33) Palese, P., Tobita, K., Ueda, M., and Compans, R.W.: Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology*, 61, 397–410 (1974).
34) Shaw, M.W., Choppin, P.W., and Lamb, R.A.: Previously unrecognized influenza B virus glycoprotein from bicistronic mRNA that also encodes the viral neuraminidase. *Proc. Natl. Acad. Sci. USA*, 80, 4879–4883 (1983).
35) Williams, M.A. and Lamb, R.A.: Determination of the orientation of an integral membrane protein and sites of glycosylation by oligonucleotide-directed mutagenesis: Influenza B virus NB glycoprotein lacks a cleavable signal sequence and has an extracellular NH_2-terminal region. *Mol. Cell. Biol.*, 6, 4317–4328 (1986).
36) Bos, T.J., and Nayak, D.P.: Identification of defects in the neuraminidase gene of four temperature-sensitive mutants of A/WSN/33 influenza virus. *Virology*, 154, 85–96 (1986).
37) Breuning, A., Muller, K. and Scholtissek, C.: Mutants of an influenza A reassortant which are cold-sensitive (cs) as well as temperature-sensitive (ts): On the role of the neuraminidase activity for influenza virus infection. *Virology*, 156, 101–106 (1987).
38) Breuning, A., and Scholtissek, C.: A reassortant between influenza A virus (H7N2) synthesizing an enzymatically inactive neuraminidase at 40°C which is not incorporated into infectious particles. *Virology*, 150, 65–74 (1986).
39) Colman, P.M.: Neuraminidase: Enzyme and antigen. In *The influenza virus*, edited by Krug, M.K., pp.175–210 (1989). Plenum Press, New York.
40) Burmeister, W.P., Ruigrok, R.W. and Cusack, S.: The 2.2 A resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. *EMBO J.*, 11, 49–56 (1992).
41) Yamamoto-Goshima, F., Maeno, K., Shibata, S., Mano, H., Suzuki, T. and Nishi, Y.: Analysis of the mechanism of influenza B virus inactivation by guinea pig serum. *Arch. Virol.*, 103, 275–282 (1988).
42) Yamamoto, F., Maeno, K., Shibata, S., Iinuma, M., Miyama, A. and Kawamoto, Y.: Inactivation of influenza B virus by normal guinea-pig serum. *J. Gen. Virol.*, 68, 1136–1141 (1987).
43) Krizanova, O. and Rathova, V.: Serum inhibitors of myxoviruses. *Current Topics in Microbiolgy and Immunology*, 47, 125–151 (1969).
44) Chu, C.M.: The action of normal mouse serum on influenza virus. *J. Gen. Microbiol.*, 5, 739–757 (1951).
45) Cooper, N.R.: The role of the complement system in host defense against virus diseases. In *Concepts in viral pathogenesis*, edited by Notkins, A.L. and Oldstone, M.B.A., pp.20–25 (1984). Springer-Verlag: New York, Berlin, Heidelberg, & Tokyo.