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Development of Protease Activation Mutants of HVJ (Sendai Virus) in Persistently Infected Cell Cultures

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SUMMARY

HVJ wild-type virus, in which the F protein is activated by trypsin but not by elastase, was spontaneously converted to a mutant with an F protein characterized by being activated by elastase alone. This spontaneous mutation generally occurred during serial passages of cells persistently infected with HVJ, even though the cells were first established by infection with plaque-purified wild-type virus. Multiple-cycle replication, plaque formation, haemolysis and SDS–polyacrylamide gel electrophoretic (SDS–PAGE) analysis showed that all the elastase-activated mutants isolated from HVJ carrier cells no longer required trypsin for F protein activation. At early passages, these protease activation mutants did not show temperature-sensitive (*ts*) growth, while at a later stage the mutants, together with the *ts* mutation, appeared dominant. The frequency of such a protease activation mutation during passage in the HVJ carrier cells seemed to depend on the cell species, but was increased when compared to lytic infections.

INTRODUCTION

Some altered characteristics of paramyxoviruses derived from persistently infected cells have been studied to date in relation to the establishment and maintenance of their persistent infections. The alterations include temperature sensitivity (Preble & Youngner, 1972; Kimura *et al.*, 1975; Ju *et al.*, 1978), weak cytopathogenicity (Kimura *et al.*, 1975; Wechsler *et al.*, 1979), altered mobility of structural proteins (P, HN, NP and M) on SDS–PAGE (Kimura *et al.*, 1979; Yoshida *et al.*, 1979; Wechsler *et al.*, 1979; Ogura *et al.*, 1981*b*) etc. Since the F protein, which is one of the two types of spikes on the envelope of the viruses, is generally thought to play the essential role in the initiation of infection, it seems likely that some biological variations in the F protein may also occur during persistent infection.

The F glycoprotein of paramyxoviruses cleaved by proteolysis of F_0 into two disulphide-linked fragments, F_1 and F_2, is well known to induce biological activities such as cell fusion, haemolysis and infectivity (Homma & Ohuchi, 1973; Scheid & Choppin, 1974; Shimizu *et al.*, 1974; Hightower *et al.*, 1975; Nagai *et al.*, 1976*a*, *b*; Scheid *et al.*, 1978). Moreover, the F protein is thought to be immunologically and pathologically responsible for essential functions in paramyxovirus infection. The cell-fusing activity of the virus makes possible spread of infection from cell to adjacent cell by fusion of their plasma membranes, so that antibody to the F glycoprotein completely prevents the spread of infection (Merz *et al.*, 1980). Particularly, susceptibility of Newcastle disease virus (NDV) glycoproteins to proteolytic activation has recently proved to be an important factor in determining the pathogenicity of the virus (Nagai *et al.*, 1976*a*, 1979; Garten *et al.*, 1980). In HVJ, protease activation mutants, which differ from the wild-type virus with regard to the specific protease

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required for activation of biological properties, have already been isolated by treatment of the virus with nitrous acid *in vitro* (Scheid & Choppin, 1976).

In this communication, we describe the isolation and studies of the frequency of occurrence of HVJ protease activation mutants from persistently infected cell cultures, and discuss the biological significance of such a mutation in the F protein during the maintenance of virus persistent infection.

METHODS

Virus. The Nagoya-1-60 strain of HVJ was used in all experiments. HVJcl.01 was obtained by three successive isolations of plaques of the wild-type virus at 39 °C. Stock virus of HVJcl.01 was propagated by allantoic inoculation of 10-day-old embryonated eggs with 0·2 ml of 5×10^3 p.f.u. After incubation at 39 °C for 3 days, allantoic fluids were harvested and stored at −90 °C. All elastase-activated mutants were grown in LLCMK2 cells in the presence of 5 μg/ml elastase. Culture media were harvested and stored at −90 °C.

Cell cultures and media. GM2 (a continuous cell line derived from 3-methylcholanthrene-induced fibrosarcoma of an inbred golden hamster; Ogura *et al.*, 1981*a*), Vero, LLCMK2 cells and their HVJcl.01 carrier cells were used in this study. The other HVJ carrier cells, G2-HVJ and THEL-HVJ cells (Ogura *et al.*, 1981*b*) were also used in the present experiment. Vero and LLCMK2 cells were cultured at 34 °C in Eagle's minimum essential medium (MEM) containing 2% and 5% calf serum respectively. The other cells were grown at 34 °C in Eagle's MEM supplemented with 10% calf serum. The maintenance medium consisted of serum-free Eagle's MEM.

Haemagglutinin titrations. Haemagglutinin was titrated by a microtitre technique according to the procedure of Sever (1962).

Plaque assays. These were done in LLCMK2 cells in 35 × 10 mm plastic dishes with 4 ml overlay containing equal parts of 2 × concentrated Eagle's MEM and 2% purified agar (Difco). Proteases, 1 μg/ml acetyltrypsin or 5 μg/ml elastase were added to the overlay.

Multiple-cycle replication. LLCMK2 monolayers ($1·5 \times 10^6$ to $3·0 \times 10^6$ cells/dish) were infected with 5×10^3 p.f.u. or 2×10^4 p.f.u. of virus and incubated in maintenance medium at 32 °C. Haemagglutinin released into culture media was assayed at the 5th or 7th day, and again on the 14th day after infection.

Haemolysis assays. Assays were done according to the method of Scheid & Choppin (1974).

Isotopic labelling and virus purification. Confluent monolayers of LLCMK2 cells were inoculated with HVJcl.01 or elastase-activated mutants at an input multiplicity of infection of approx. 5. After 24 h, the infected cells were labelled with 25 μCi/ml [^{3}H]glucosamine in maintenance medium containing 10 mM-fructose instead of glucose. At 72 h after infection the culture media were harvested and centrifuged at 8000 rev/min for 30 min to remove cell debris. The virus was then concentrated by centrifugation at 25000 rev/min in a Beckman SW27.1 rotor for 90 min. Following this the virus was centrifuged through a 10 to 40% (w/w) linear sucrose gradient (in 5 mM-tris–HCl, 1 mM-EDTA pH 7·4) for 45 min in a Beckman SW27.1 rotor. The virus band was collected, diluted with phosphate-buffered saline (PBS), and sedimented. The resulting pellet was resuspended in PBS.

SDS–PAGE. This was done by the procedure of Laemmli (1970), using slabs of 1 mm thickness containing 10% polyacrylamide. Detection of [^{3}H]glucosamine was performed by autofluorography using En^{3}Hance (New England Nuclear). The autofluorogram was recorded on Kodak X-Omat RP film.

Assays for mutation frequency. In order to measure numbers of elastase-activated mutants in a sample, samples at limit dilution [usually 10^2 to 10^4 p.f.u. (titres in the presence of trypsin)] were pretreated with elastase and inoculated on to LLCMK2 cell monolayers in

dishes. Multiple-cycle replication was carried out in the presence of 5 μg/ml elastase at 32 °C. From a ratio of numbers of dishes in which haemagglutinin was detected to total dishes (usually 10 to 30 dishes) inoculated with limitedly diluted samples, the mutation frequency according to virus dilution was calculated. As another method, plaque formation was also done in the presence of elastase. In comparison with detectable sensitivity for elastase-activated mutants, however, multiple-cycle replication was shown to be better than plaque formation by one to five times. Therefore, mutation frequency presented in this paper was measured by multiple-cycle replication. Over 10^5 p.f.u. of virus as inocula were not tested because there was a possibility that many of the cells were infected by the initial inoculum. Therefore, with a relatively higher concentration of inoculum, such as 5×10^4 p.f.u. of virus, increased numbers of dishes for inoculation were employed.

Chemicals and isotope. Acetylated trypsin, elastase, 2 × crystallized and *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK) were obtained from Sigma. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was purchased from Nakarai Chemicals, Japan. D-[6-^{3}H]glucosamine–HCl (38 Ci/mmol) was obtained from Amersham International.

RESULTS

Isolation of protease activation mutants from culture media of HVJ carrier cells

HVJcl.01, purified by three successive isolations of plaques formed on LLCMK2 cells incubated at 39 °C, was previously confirmed to be activated by certain kinds of proteases. Multiple-cycle replication, plaque formation and haemolysis showed that HVJcl.01 required trypsin, but not elastase, for activation of its biological properties. The F_0 protein of the virus was confirmed to be cleaved by trypsin, but not by elastase on SDS–PAGE as shown later in Fig. 3.

GM2, Vero and LLCMK2 cells were infected with HVJcl.01 at an input multiplicity of infection of approx. 0·01. After the 5th to 8th passage, c.p.e. appeared and some cells were destroyed. The residual cells, which almost all expressed HVJ antigens both on the membrane and in the cytoplasm, continued to be cultured at 34 °C. At an appropriate passage number, culture media of confluent monolayers were replaced with serum-free maintenance media and they were harvested after 24 h incubation. These culture media whose infectivities were adjusted to 2×10^4 p.f.u. (titres in the presence of trypsin) were pretreated with trypsin or elastase at room temperature for 30 min and then inoculated into LLCMK2 cells. After 1 or 2 weeks incubation at 32 °C in maintenance media in the presence or absence of trypsin or elastase, haemagglutinin in the culture media was assayed.

As shown in Table 1, GM2-HVJcl.01 cells produced protease activation mutants which were activated by elastase (HVJpi-e) at the 12th, 31st, 50th and 100th passage. HVJpi-e was detected also in the culture media of Vero-HVJcl.01 cells at the 50th passage and LLCMK2-HVJcl.01 cells at the 31st and 50th passage. In the culture media of G2-HVJ and THEL-HVJ cells which have been passaged *in vitro* for over 12 years, no elastase-activated mutants were detected in spite of several trials.

Cloning of elastase-activated mutants and reconfirmation of their activation by elastase

From culture media of GM2-HVJcl.01 cells at the 12th and 50th passage, nine (HVJpi-e cl.1201 and 1202, cl.1204 and 1205 and cl.1251 to 1255) and five (HVJpi-e cl.5001 to 5005) mutants respectively were cloned by three successive isolations of plaques and further examined for susceptibility to proteolytic activation. Multiple-cycle replication (Table 2) and plaque formation (Fig. 1) demonstrated that all clones were susceptible to elastase, and no longer to trypsin. They did not show any haemolytic activity by treatment with various concentrations of trypsin. However, in contrast to the wild-type, appropriate concentrations

Table 1. *Multiple-cycle replication of HVJcl.01 and HVJ released from various HVJ carrier cultures in the presence of specific proteases*

Culture* medium from	In the presence of	Yield (HAU/0·025 ml) at day 7/day 14 at passage number 0	12	31	50	100	>450
GM2-HVJcl.01	No proteases		<1/<1	<1/<1	<1/<1	<1/<1	
	Trypsin (1 μg/ml)		16/4	16/16	32/64	8/64	
	Elastase (5 μg/ml)		<1/64	<1/16	<1/1	<1/4	
Vero-HVJcl.01	No proteases		<1/<1	<1/<1	<1/<1		
	Trypsin (1 μg/ml)		8/32	8/16	8/8		
	Elastase (5 μg/ml)		<1/<1	<1/<1	<1/1		
LLCMK2-HVJcl.01	No proteases		<1/<1	<1/<1	<1/<1		
	Trypsin (1 μg/ml)		32/32	16/32	16/32		
	Elastase (5 μg/ml)		<1/<1	<1/1	<1/1		
G2-HVJ	No proteases						<1/<1
	Trypsin (1 μg/ml)						16/32
	Elastase (5 μg/ml)						<1/<1
THEL-HVJ	No proteases						<1/<1
	Trypsin (1 μg/ml)						8/32
	Elastase (5 μg/ml)						<1/<1
Egg-grown HVJcl.01	No proteases	<1/<1					
	Trypsin (1 μg/ml)	64/8					
	Elastase (5 μg/ml)	<1/<1					

* LLCMK2 cells were infected with 2×10^4 p.f.u. of virus (titre in the presence of trypsin) which was contained in the culture media. Inoculum was treated with 1 μg/ml trypsin and 5 μg/ml elastase prior to inoculation. Infected cells were incubated at 32 °C.

Table 2. *Multiple-cycle replication of cloned HVJpi-e in LLCMK2 cells in the presence of specific proteases*

Inoculum†	Yield* (HAU/0·025 ml) in the presence of: No proteases	Trypsin (1 μg/ml)	Elastase (5 μg/ml)
HVJpi-e cl.1201	<1	<1	32
cl.1202	<1	<1	32
cl.1204	<1	<1	64
cl.1205	<1	<1	32
cl.1251	<1	<1	4
cl.1252	<1	<1	16
cl.1253	<1	<1	32
cl.1254	<1	<1	16
cl.1255	<1	<1	16
cl.5001	<1	<1	32
cl.5002	<1	<1	16
cl.5003	<1	<1	64
cl.5004	<1	<1	32
cl.5005	<1	<1	64
HVJcl.01	<1	64	<1

* Virus yields were determined after 5 days incubation at 32 °C.
† LLCMK2 cells were inoculated with 5×10^3 p.f.u. of virus.

of elastase activated their haemolysis (Fig. 2). By SDS–PAGE analysis using [^{3}H]glucosamine-labelled HVJpi-e, F_0 protein of these clones proved to be cleaved into F_1 by treatment with elastase, but not with trypsin (Fig. 3). These results clearly indicate that all the clones are elastase-activated mutants.

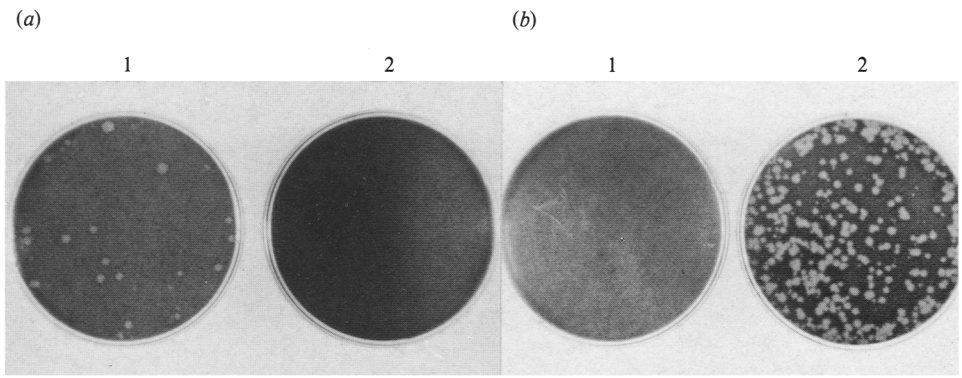

Fig. 1. Requirement for specific proteases for plaque formation in LLCMK2 cell monolayers by (*a*) HVJcl.01 and (*b*) HVJpi-e cl.5003. Cell cultures were incubated at 32 °C for 10 days after inoculation of virus in the presence of 1 μg/ml trypsin (1) or 5 μg/ml elastase (2).

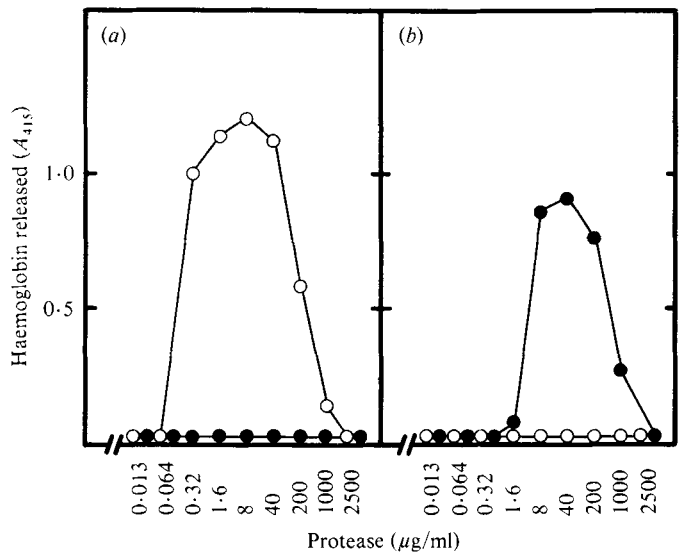

Fig. 2. Susceptibility of (*a*) HVJcl.01 and (*b*) HVJpi-e cl.5003 to the activation of haemolytic activity by proteases. Purified virus grown in LLCMK2 cells was incubated for 10 min at 37 °C with trypsin (○) or elastase (●) at the various concentrations. The elastase used was treated with *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK). Aliquots were then assayed for haemolysing activity.

Relation to temperature-sensitive (ts) *mutation*

It is well known that paramyxovirus persistently infected cells produce *ts* mutants during continuous passage (Preble & Youngner, 1973; Ju *et al.*, 1978). Therefore, in order to determine the possible existence of *ts* mutants in the above HVJpi-e, the ratio of virus yield of the cloned respective HVJpi-e at 39 °C to those at 32 °C was examined (Fig. 4). Although all HVJpi-e clones isolated from the culture medium at the 50th passage were *ts* mutants, various grades of temperature sensitivity were found to exist among HVJpi-e clones from the culture medium of the 12th passage. These results show protease activation mutation can occur independently of *ts* mutation.

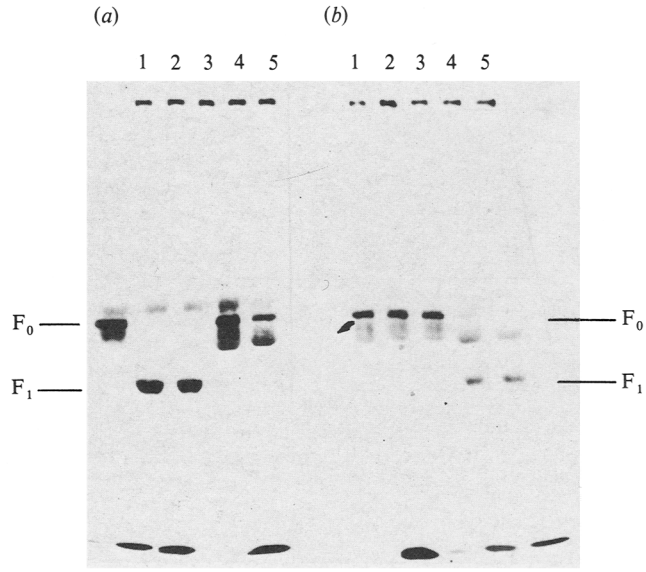

Fig. 3. Cleavage of F_0 glycoprotein of (*a*) HVJcl.01 and (*b*) HVJpi-e cl.5003 by trypsin or elastase. [^{3}H]glucosamine-labelled virions grown in LLCMK2 cells were incubated with no proteases (1), 4 μg/ml (2) or 8 μg/ml (3) trypsin and 20 μg/ml (4) or 40 μg/ml (5) elastase at 37 °C for 10 min. All these samples were analysed by 10% polyacrylamide gel electrophoresis and autofluorography.

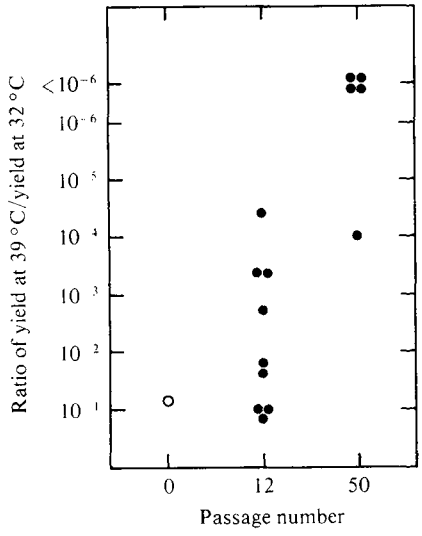

Fig. 4. Temperature sensitivity of HVJcl.01 (○) or HVJpi-e (●). LLCMK2 cell monolayers were infected with virus at an input multiplicity of infection of 5 and incubated at 32 °C and 39 °C for 72 h. The culture media were harvested and the infectivities were assayed at 32 °C in the presence of trypsin for HVJcl.01 or elastase for HVJpi-e. Ratio of yield at 39 °C to yield at 32 °C was plotted.

Frequency of elastase activation mutation in persistent infection

The frequency of protease activation mutation in lytic infection in the presence or absence of MNNG was first measured to be compared with that in persistent infection. GM2, Vero and LLCMK2 cells were infected with HVJcl.01 at an input multiplicity of infection of

Table 3. *Mutation frequency of elastase-activated mutants in culture media of lytically or persistently HVJcl.01-infected cells*

	In the presence of MNNG (μg/ml)			
(*a*) Lytically infected with HVJcl.01*	0	2·5	5	10
GM2	$7{\cdot}1 \times 10^{-6}$	$1{\cdot}4 \times 10^{-5}$	$2{\cdot}8 \times 10^{-5}$	$3{\cdot}6 \times 10^{-5}$
Vero	$<6{\cdot}7 \times 10^{-7}$	$6{\cdot}7 \times 10^{-6}$	$3{\cdot}6 \times 10^{-5}$	$2{\cdot}1 \times 10^{-5}$
LLCMK2	$<6{\cdot}7 \times 10^{-7}$	$3{\cdot}3 \times 10^{-6}$	$4{\cdot}2 \times 10^{-6}$	$1{\cdot}9 \times 10^{-5}$
	Passage number			
(*b*) Persistently infected with HVJcl.01†	12	31	50	100
GM2-HVJcl.01	$2{\cdot}5 \times 10^{-4}$	$5{\cdot}0 \times 10^{-5}$	$1{\cdot}0 \times 10^{-4}$	$1{\cdot}0 \times 10^{-4}$
Vero-HVJcl.01	$<1{\cdot}0 \times 10^{-5}$	$1{\cdot}0 \times 10^{-5}$	$2{\cdot}5 \times 10^{-5}$	NT‡
LLCMK2-HVJcl.01	$<1{\cdot}0 \times 10^{-5}$	$5{\cdot}0 \times 10^{-5}$	$1{\cdot}0 \times 10^{-4}$	NT

* Cell monolayers were infected with HVJcl.01 at an input multiplicity of infection of 5 and incubated in serum-free media at 34 °C in the absence or presence of MNNG. After 72 h, culture media were harvested and assayed for mutation frequency by multiple-cycle replication.

† Cell monolayers were incubated in serum-free media at 34 °C. After 24 h, culture media were harvested and assayed for mutation frequency by multiple-cycle replication.

‡ NT, Not tested.

approx. 5. After incubation at 34 °C for 72 h in the presence or absence of MNNG (2·5, 5 and 10 μg/ml), culture media were harvested and assayed for mutation frequency.

Spontaneous mutation of elastase activation in lytic infection with HVJcl.01 occurred more frequently in GM2 cells than in Vero and LLCMK2 cells, showing its dependency on infected cell species. Treatment of the lytically infected cells with MNNG enhanced the frequency of its occurrence from 5 to 30 times (Table 3*a*).

In persistent infection, maintenance media, in which confluent monolayers were incubated at 34 °C for 24 h, were examined. GM2-HVJcl.01 cells produced elastase-activated mutants more frequently than the other carrier cultures (Table 3*b*), in accordance with the results of the spontaneous mutation in lytic infection. Generally, the frequencies in persistent infection were found to be higher than those in lytic infection and almost equal to those of lytic infection in the presence of MNNG.

On the other hand, revertants of elastase-activated mutants grown in LLCMK2 cells appeared spontaneously at frequencies of about 10^{-6} (data not shown).

DISCUSSION

Paramyxoviruses isolated from persistently infected cell cultures have been found to show temperature-sensitive growth, weak cytopathogenicity, variations in apparent mol. wt. of structural proteins etc. It is still unclear, however, what kind of biochemical variations or changes of biological functions the above alterations are linked to, and what the essential factor(s) is for persistence of the virus in cell cultures. Meanwhile, biological and biochemical characteristics of the F protein of HVJ have become clearer than those of the other virus structural proteins. It ought to be easy to select and isolate a protease activation mutant that is activated by a different protease from the one required by the parent virus.

First, newly established persistent infections in various cell lines with HVJ wild-type virus purified by plaque isolations at 39 °C were designed to obtain mutants from them. These new persistently infected cells were shown to produce elastase-activated mutants in the culture media. The altered protease sensitivity of the F protein of these cloned mutants was confirmed by a series of experiments such as multiple-cycle replication, plaque formation, haemolysis

and SDS–PAGE. All of them were found to lose concomitantly susceptibility to trypsin. This is different from the report that the majority of the elastase-activated mutants were activated by either elastase or trypsin (Scheid & Choppin, 1976). The present study, moreover, showed increased frequency of the mutation in persistent infection, compared with that of lytic infection in the same cells.

As long as HVJ is grown by passage in embryonated eggs in which chorioallantoic fluids contain a trypsin-like protease different from elastase or chymotrypsin (Muramatsu & Homma, 1980), mutants activated by either elastase or chymotrypsin multiply poorly (Scheid & Choppin, 1976) and may be naturally excluded even though these mutants spontaneously occur. These circumstances seem to differ greatly from the above case of persistent infection *in vitro*.

Recent evidence has demonstrated that strain differences in proteolytic cleavability of virus glycoproteins may account for the variations in pathogenicity of NDV in terms of the ability of the virus to kill chick embryos as well as the speed with which this killing occurs (Nagai *et al.*, 1976*a*, 1979; Garten *et al.*, 1980). This virus is cleaved intracellularly by endogenous cell proteases (Nagai *et al.*, 1976*b*; Seto *et al.*, 1981). In contrast to NDV, cleavage of F_0 protein of HVJ occurs extracellularly on the plasma membrane, or soon after release from infected cells (Lamb *et al.*, 1976; Seto *et al.*, 1981). HVJ belonging to parainfluenza type 1 causes respiratory infections in mice (van Nunen *et al.*, 1967; Robinson *et al.*, 1968; Appell *et al.*, 1971). Mutant HVJ resistant to trypsin cleavage showed no pathogenicity in mouse lung, although its growth was only single-cycle (Tashiro & Homma, 1980). This means that trypsin-like protease is usually secreted in the respiratory organs of mice so that the original HVJ capable of activation by trypsin can undergo multiple-cycle growth. Thus, the susceptibility of the F glycoprotein of HVJ to proteolytic enzymes might play a prime role in determining the organ tropism, or tissue tropism as mentioned also by Scheid & Choppin (1976), and seems to be paralleled with pathogenicity at the organ or tissue level, but not at the cellular level, through spread of paramyxovirus infection caused by the cell-fusing activity of activated F protein (Merz *et al.*, 1980).

Some paramyxoviruses are known to cause persistent infection in susceptible animals and man. Measles virus, for example, has been implicated in the slowly progressive neurological disease, subacute sclerosing panencephalitis (Morgan & Rapp, 1977). Pathogenesis of HVJ infection to the central nervous system (Shimokata *et al.*, 1976) and the inner ear (Shimokata *et al.*, 1977) of mice was also found by intracerebral inoculation with the virus. However, it is unclear whether or not the inoculated virus could undergo multiple cycles of infection in those tissues. Whether protease activation mutants may grow in multiple cycles and cause diseases in organs other than respiratory tract still remains to be clarified. This is an interesting and important problem for future research on HVJ pathogenesis. Thus, the spontaneous occurrence *in vitro* of protease activation mutants and their increased frequency in HVJ persistent infection might suggest the possible acquisition of pathogenicity for some organs that are not susceptible so far, or the possibility of virus persistence in the original target organs.

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