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Review Article

VIRAL PATHOGENESIS: MECHANISM OF ACUTE AND PERSISTENT INFECTIONS WITH PARAMYXOVIRUSES

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INTRODUCTION

Paramyxoviruses are a group of infectious agents that cause a variety of diseases in man and animals, such as mumps, measles, respiratory infections in infants, Newcastle disease in chickens, and canine distemper. Besides these acute diseases, the viruses of this group are known to often induce long-term persistent infections and have long been suspected to be involved in chronic neurological diseases such as subacute sclerosing panencephalitis and multiple sclerosis.

Viral pathogenesis, the way in which a virus produces a disease, involves a complex interaction between the virus and the infected organism. Thus, it is, in general, not easy to define the molecular basis for pathogenesis with any given virus.

A virus is pathogenic for a particular host if it can infect the host and produce signs and symptoms of disease. A virus strain is said to be more virulent than another if it regularly produces more severe signs or disease in a host in which both strains are pathogenic. Thus, one way to study such a difficult problem as pathogenesis is to define the factors responsible for the differences in virulence. In this context, Newcastle disease virus (NDV) provides a good research model since it comprises a wide variety of well-characterized strains which differ markedly in virulence but are indistinguishable from each other by conventional serology.

The processes of diseases in long-term persistent infections are even more complicated. Therefore, efforts have mainly concentrated on elucidation of the mechanism of induction of persistent infections in cultured cells. Of the paramyxoviruses, Sendai virus (hemagglutinating virus of Japan, HVJ) has probably been best studied in this respect.

In this article, we illustrate our recent attempts to answer the questions related to acute and persistent infections with NDV and Sendai virus as well as those reported by other authors for different viruses.

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GENERAL FEATURES OF PARAMYXOVIRUSES

Most of information on the suructure, function and replication of paramyxovirus has been obtained from Newcastle disease virus (NDV), Sendai virus and Simian virus 5 (SV5) (see reviews, 1, 2, 3, 4, 5, 6).

The virion consists of a helical ribonucleoprotein, the nucleocapsid, which is surrounded by an envelope. The nucleocapsid is composed of a single-stranded RNA genome with approximately 15,000 bases, the major subunit protein (NP) and two other polypeptides, L and P. The genome RNA possesses antimessage polarity, *i.e.*, negative sense. On this negative strand, the viral RNA transcriptase initiates transcription at a single promotor to yield 6 to 8 virus-specific complementary messenger ("positive strand") molecules. Recently, we demonstrated that both L and P proteins are associated with the transcriptive complex isolated from infected cells and that both are required to reconstitute *in vitro* a transcription system with a functional template.[7] It remains to be elucidated whether the transcriptase synthesizes a long poly-cistronic m-RNA which is subsequently processed into monocistronic messages or whether the transcription stops and restarts at each junction between message sequences.

After transcription and protein synthesis, there is a switch to the synthesis of full-length positive strands which serve as templates for the synthesis of full-length negative strands of progeny virus (replication). When inhibitors of protein synthesis were added to the virus-infected cells and the species of virus-specific RNA were analyzed, it was found that m-RNAs continued to be synthesized for hours, whereas synthesis of full-length RNA was rapidly inhibited.[8] Therefore, protein(s) involved in the replication of viral genome is relatively unstable or should be continuously supplied, in contrast to the transcription which continues for hours utilizing already synthesized pools of transcriptase. However, detailed information on the mechanisms modulating the transcriptive and replicative processes of paramyxoviruses is yet unavailable.

The envelope contains lipids which are derived from the plasma membrane of host cells and are present in the form of a bilayer. The outer side of the bilayer exhibits protrusions, spikes which are composed of glycoproteins, and the inner side is coated with carbohydrate-free membrane (M) protein. There are two types of spikes; one consists of glycoprotein HN, which has hemagglutinating and neuraminidase activities,[9, 10] and the other consists of glycoprotein F, which induces cell fusion and hemolysis.[11, 12] Both glycoproteins play essential roles in the initiation of infection; glycoprotein HN is responsible for the attachment of the virus to neuraminic acid-containing receptors on the host cell surface (Fig. 1A), whereas glycoprotein F is involved in the subsequent penetration process which is mediated by fusion of the viral envelope with the cellular plasma membrane (Fig. 1B). Thus, the uncoating, the transfer of viral genome into cytosol, takes place directly through fusion at the plasma membrane. This is a feature of paramyxoviruses distinctive from those of most other enveloped RNA viruses such as Semliki forest, vesicular stomatitis and influenza viruses.[13] These viruses are, after attaching to the cell surface, internalized by endocytosis and sequestered into the intracellular vacuoles and lysosomes. Fusion activity of these viruses is expressed at low but not neutral pH, and the low pH in the lysosomes triggers fusion between the viral envelope and lysosomal membrane, thus releasing the nucleocapsids into cytosol.[14, 15, 16, 17]

Envelope maturation is a multi-step process involving sequential incorporation of viral proteins into cellular membranes (Fig. 2). Fractionation studies of cells infected with NDV[18, 19] and Sendai virus[20] have shown that the polypeptide chains of the glycoproteins are synthesized on the rough endoplasmic reticulum (step 1 in Fig. 2). From there they migrate via the Golgi apparatus to the plasma membrane (steps 2-5). The M protein presumably

synthesized on both membrane-bound and membrane-free polysomes[21] becomes attached to the areas of plasma membrane containing the glycoproteins (step 6), which is folllowed by the formation of a patch of virus-specific membrane, the immediate precursor for the envelope (step 7).[19] Neither the patch formation nor the association of nucleocapsid with the plasma membrane (step 8) seems to occur when M protein synthesis is selectively suppressed in cells infected with a temperature-sensitive mutant of Sendai virus.[22] These lines of evidence suggest a crucial role of M protein in the virus assembly. This important role of M protein has also been emphasized by an *in vitro* reconstitution study showing that the glycoproteins and the nucleocapsids do not form a complex unless M protein is present in the reaciton mixture[23] as well as by a chemical cross-linking analysis of proteins in intact virions.[24] The mature virus particle is then released by budding (step 9), but the mechanism involved in the initiation of budding remains to be elucidated. The sequential events outlined above were further suggested by recent studies by immunoelectron microscopy[21] and by the use of monensin, an inhibitor of intracellular transport of glycoproteins (manuscript in preparation).

During the course of intracellular migration, the glycoproteins undergo posttranslational modifications. These involve sequential glycosylation and proteolytic cleavage. As will be shown later, the proteolytic cleavage is of great importance for the expression of biological activities of the glycoproteins. Whether the proteolytic cleavage occurs or not depends upon both the virus and the host cell, but the cleavage is not necessary for virus assembly.[11, 12, 25] Thus the spikes of virions consist of either uncleaved precursor glycoproteins or the cleavage products. As shown in Fig. 3, the fusion glycoprotein consists of either the precursor F_0 or its cleavage product F composed of two disulfide-bonded fragments F_1 and F_2.[19, 26] The hemagglutinin-neuraminidase spike contains either the precursor HN_0 or the HN glycoprotein which is obtained by removal of a small fragment from HN_0.[25, 27, 28] The latter precursor-product relationship has been found only with several NDV strains,[25, 29] whereas the former is found with all the paramyxoviruses so far analyzed.[30, 31, 32, 33]

MOLECULAR BASIS FOR THE VIRULENCE OF NEWCASTLE DISEASE VIRUS

NDV comprises a wide range of strains which differ markedly in virulence for their natural host, the chicken. This virus grows well and is readily titrated in certain hosts, and its virulence can be measured on a quantitative basis in chick embryo and young chickens. NDV has, therefore, long been used as a suitable model for the investigation of viral pathogenicity.[34]

1. *Virulence of NDV* Virulence of NDV can be measured in terms of the ability of the virus to kill chick embryo (mean death time, MDT), or the ability to paralyse or kill the new born chicken (intracerebral pathogenicity, IC index).[34] Table 1 shows the virulence of 5 virulent and 5 avirulent strains which have been arbitrarily chosen for the present study. The range of MDT varies from about 50 hr for virulent strains to infinity for nonlethal strains. The IC index is expressed by scoring, according to the method below, cumulatively for 8 days following intracerebral inoculation into 1-day-old chickens. A dead bird is scored as 2, one showing signs of illness as 1, and a healthy bird as zero. The sum obtained is divided by the number of observations made and hence the results may vary for a maximum of 2 (100% mortality) down to 0 (no recorded symptoms). The index runs about inversely parallel with the MDT (Table 1).

2. *Strain-Dependent Differences in the Susceptibility of NDV Glycoproteins to Proteolytic Cleavage* When infected BHK-21 cells were pulse-labeled with radioactive amino acids

and chased, and subsequently subjected to polyacrylamide gel electrophoresis, three distinct patterns of the viral glycoprotein synthesis could be discriminated[25] (Table 1). The first type

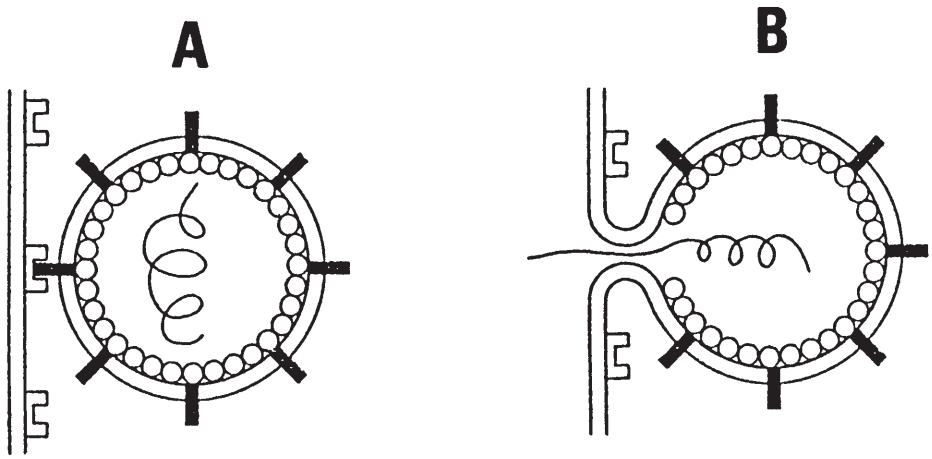

Fig. 1 Roles of Paramyxovirus glycoproteins in initiation of infection. For details see text.

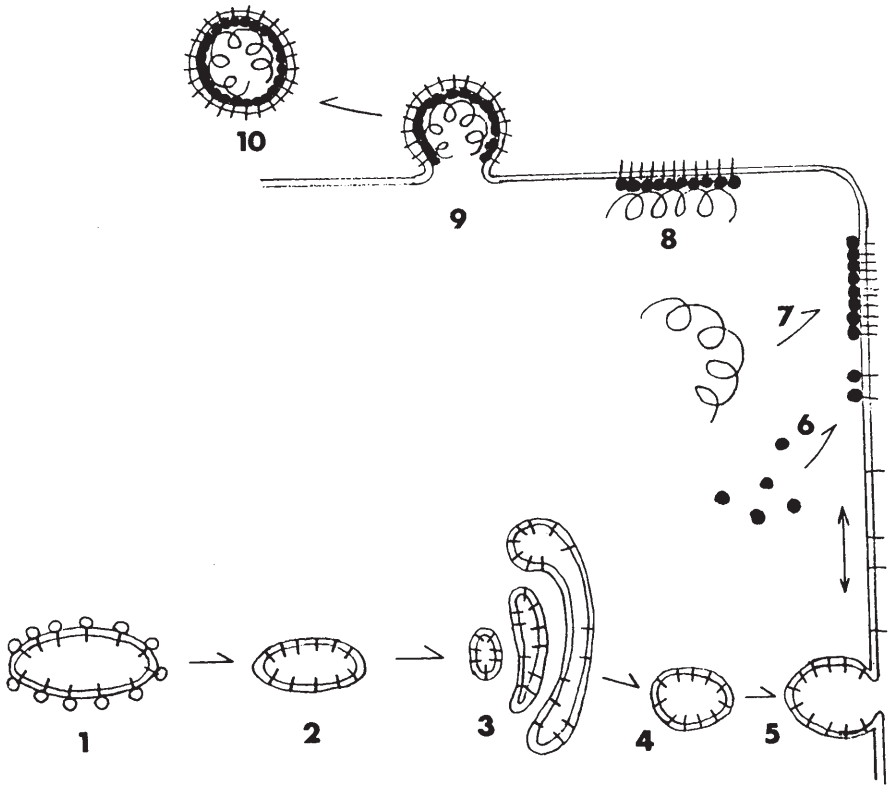

Fig. 2 Sequence of events involved in paramyxovirus assembly. For details see text.

shows an efficient proteolytic processing of F_0 precursor to yield F. In this type no HN_0 precursor is present and HN glycoprotein is detected as a direct translation product. Thus, the virions formed contain HN and F glycoproteins and this pattern is characteristic of all of the 5 virulent strains. The second type shows glycoprotein HN and the precursor glycoprotein F_0, indicating no proteolytic processing of F_0. The HN glycoprotein is a direct translation product in this case, too. This pattern is found with avirulent strains La Sota, B_1 and F. The third type is typical for avirulent strains Ulster and Queensland. The precursor F_0 is not processed and, in addition, HN_0 precursor is detected which also has not undergone processing. Thus, the infection with these viruses produces virions which contain both glycoproteins in unleaved precursor forms, HN_0 and F_0. Essentially the same variations in glycoprotein profile were observed when the viruses were grown in MDBK and primary chick embryo cells.

3. *Biological Activities of Viral Glycoproteins and Their Dependence on Proteolytic Cleavage* The cleavage of the precursors of NDV glycoproteins is necessary for their biological activities[25, 27] as previously found with glycoprotein F of Sendai virus.[11, 12] This is shown by an example of Ulster strain which has been grown in MDBK cells and has both glycoproteins in precursor forms (Table 2). When the virions were subjected to trypsin treatment *in vitro*, both precursors were cleaved to yield HN and F, respectively, and their biological activities, hemagglutination and neuraminidase of HN, and membrane fusing capacity (hemolysis) of F, were activated. The activation of hemagglutination and neuraminidase were relatively low, if compared with that of fusion activity. This could be explained by the fact that the virions contained a fair amount of cleaved HN already before enzyme treatment. In fact, trypsin treatment of HN_0 itself isolated from the virions resulted in activation of hemagglutination and neuraminidase with factors, 30 and 15, respectively, and thus HN_0 is biologically inactive.[27] The other proteases used here (chymotrypsin, elastase, thermolysin) were effective only in proteolytic activation of HN_0 (Table 2), but not in that of F_0 (Table 2). Since both glycoproteins must be active to initiate infection (Fig. 1), the infectivity is fully expressed only when trypsin is used (Table 2). Another group of avirulent strains, La Sota, B_1 and F, also requires trypsin to be infectious, since one of the glycoproteins is present in the form of inactive precursor (F_0), whereas all the virulent strains containing both glycoproteins in cleaved forms are fully infectious before the enzyme treatment (Table 1).

4. *Differences in Host Range of Virulent and Avirulent Strains as Determined by the Susceptibility of the Viral Glycoproteins to Cleavage* The inability of the avirulent strains to yield biologically active progeny virus is a host-specific phenomenon. Infection with avirulent strains in certain hosts such as the allantoic cavity of chick embryo results in the production of active and infectious virus containing cleaved glycoproteins. These host-specific differences are probably due to the presence or absence of appropriate proteases. From the results of cleavability in a variety of hosts, the following conclusions can be drawn as summarized in Fig. 4. Only limited hosts are permissive for avirulent strains; *i.e.*, they produce virions which contain cleaved glycoproteins and thus are highly infectious and can undergo successive multiple replication cycles. Most other host systems are nonpermissive for these viruses; they produce noninfectious virus with uncleaved glycoproteins. Thus, the virus cannot spread by multiple replication cycles in these hosts unless it is activated artificially by trypsin added to the culture medium. In contrast, all the hosts so far examined are permissive for virulent strains, always yielding virions with cleaved glycoproteins and thus with capability to spread by successive replication cycles. Therefore, it has been demonstrated that the difference in host range between virulent and avirulent strains is determined by susceptibility of the viral glycoproteins to proteolytic cleavage.

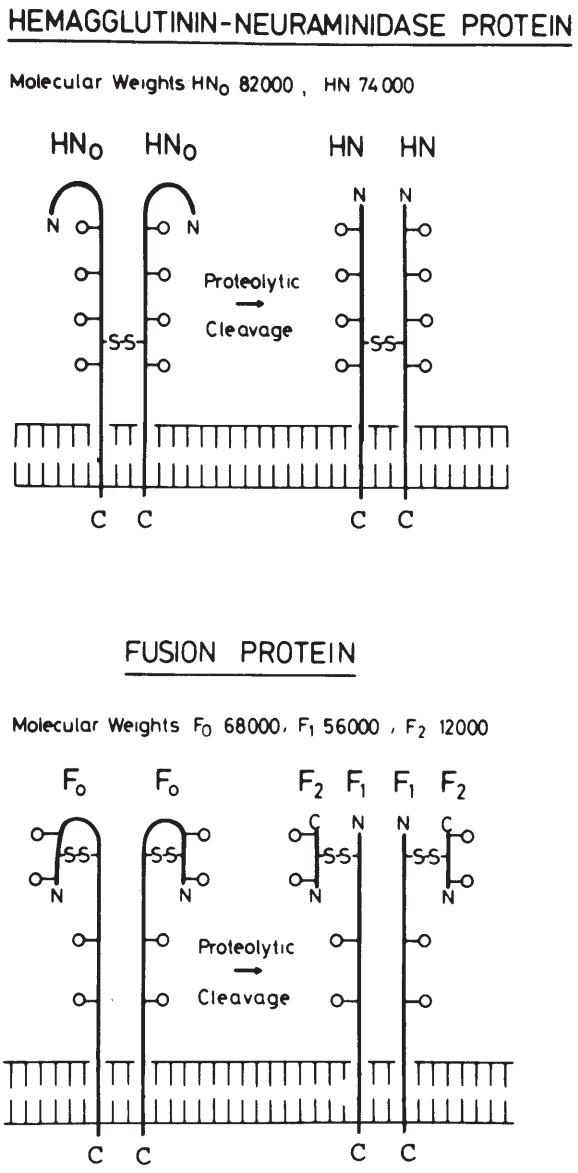

Fig. 3 The structure of the glycoproteins of Newcastle disease virus. The cleaved and uncleaved forms of the glycoproteins are shown. Each spicke contains a minimum of two glycoproteins, but there may be more. The number of carbohydrate side chains (—O) is also tentative. The disulfide bridge in the HN spike is not present in all NDV strains.

Table 1. Glycoprotein Composition and Trypsin Requirement for Infectivity of 10 Different Strains of NDV Grown in BHK-21 Cells[a]

Strain	Virulence	MDT[b]	IC index[c]	Glycoprotein composition		Trypsin-requirement[d]
Italien	Virulent	50	1.88	HN	F	−
Herts	Virulent	49	1.86	HN	F	−
Field Pheasant	Virulent	50	1.7	HN	F	−
Texas	Virulent	50	1.8	HN	F	−
Warwick	Virulent	50	1.7	HN	F	−
La Sota	Avirulent	103	0.19	HN	F_0	+
B_1	Avirulent	120	0-0.25	HN	F_0	+
F	Avirulent	168	0-0.25	HN	F_0	+
Queensland	Avirulent	∞	0.16	HN_0	F_0	+
Ulster	Avirulent	∞	0	HN_0	F_0	+

[a] For details see text.
[b,c] The data are those by Waterson *et al.*[34] and by Moore and Burke.[66]
[d] Trypsin-treatment is required (+) or not required (−) for the expression of full infectivity.

Table 2. Effect of Proteases on Ulster Strain of NDV Grown in MDBK Cells[a]

Protease	Glycoprotein		HA	N	HL	I (log)
none	HN_0	F_0	720	20	1.92	2.0
chymotrypsin	HN	F_0	3840	88	1.92	2.0
elastase	HN	F_0	3840	107	2.50	2.0
thermolysin	HN	F_0	3840	90	2.18	2.0
trypsin	HN	F	3840	90	34.10	5.0

[a] Purified virions of Ulster strain grown in MDBK cells were treated with various proteases (5-10 μg/ml) for 8 min at 37°C. The virions were then subjected to polyacrylamide gel electrophoresis to identify glycoprotein species or assayed for hemagglutination (HA), neuraminidase (N), hemolytic activity (HL) and infectivity (I) (for details, see ref. (27). Glycoproteins and activities changed by protease treatment are underlined.

5. *Significance of Glycoprotein Cleavage for the Viral Pathogenesis in an Intact Organism* From the above results it was assumed that infection with a virulent strain, which readily produces virus of full biological activity in a wide spectrum of cell types, spreads more rapidly in the organism than infection with an avirulent strain which has a narrow host range. This concept has been confirmed by studies using an intact organism, embryonated egg.[35]

Virulent and avirulent NDV strains were inoculated onto either the inner or outer side of the chorioallantoic membrane (CAM) of an 11-day-old chick embryo. The CAM consists of three germinal layers, the inner endodermal allantoic epithelium, the outer ectodermal chorionic epithelium and the mesodermal layer between the two epithelial layers. Thus, the infection initiates from either endodermal or ectrodermal epithelium. Depending on the virus strain and the inoculation site, four patterns of virus spread could be differentiated (Table 3). (1) Inoculation at the endodermal site of the CAM with a virulent strain resulted in generalized virus spread into the extra- and intra-embryonic tissues and in the death of the

embryo. Compared to the case of CAM described below, most other embryonic organs have a complex fine structure and are difficult to manipulate *in vitro*. This has prevented us from analyzing cleavage of the virus glycoproteins in these organs. However, since the glycoproteins of virulent strains were found to be cleaved in all cell types analyzed to date, it is reasonable to assume that efficient cleavage is important for such efficient spread of the virus in the embryo. (2) After endodemal inoculation with an avirulent strain, rapid spread of infection has been observed only at the endodermal layer, and the infection is unable to penetrate the barrier of the non-permissive mesoderm. For reasons not fully understood, infection of the endoderm does not interfere with the vital function of the embryo, which therefore survives. (3) After ectodermal inoculation with a virulent strain, extensive virus replication is observed in the ectodermal and mesodermal layers. The vivus spread in these tissues has been sufficient to kill the embryo before the virus has had a chance to spread into other tissues including the embryo. These two germinal layers contain the respiratory organ,[36] and the confinement of infection to this organ could be lethal. (4) Finally, ectodermal infection with an avirulent strain neither spreads from the inoculation site nor kills the embryo. The results of (3) and (4) suggest that the ectodermal and mesodermal layers of CAM are the targets of the fatal infection. The cleavability of the virus glycoproteins was examined in all three layers of CAM which were maintained *in vitro*. The results have demonstrated that the glycoproteins of virulent strains are cleaved in all three layers, whereas those of avirulent strains are proteolytically activated only in the endoderm (Table 3). These observations demonstrate that the susceptibility of the virus glycoproteins to proteolytic cleavage is of great importance for the spread of infection and tropism to target organs and thus for the pathogenesis by NDV.

Table 3. Spread of a Virulent and an Avirulent Strain of NDV in Embryonated Egg[a]

Tissue	Inoculation site in CAM				Glycoprotein synthesized[b]	
	Endoderm		Ectoderm			
	V	AV	V	AV	V	AV
CAM, endoderm	+4	+4	+4	0	HN, F	HN, F
mesoderm	+4	0	+4	0	HN, F	HN_0, F_0
ectoderm	+4	0	0	0	HN, F	HN_0, F_0
amnion sac	+4	0	0	0	n.t.	n.t.
yolk sac	+4	0	+1	0	n.t.	n.t.
embryo	+4	0	0	0	n.t.	n.t.
mortality (%)	100	0	100	0	—	—

[a] A virulent strain (V), Italien, and an avirulent strain (AV), Ulster, were inoculated into 11-day-old embryonated eggs and virus spreads were examined by determining the virus titers in various tissues as well as by immunofluorescence of chorioallantoic membrane (CAM). Either the endoderm or ectoderm of CAM was the site of initiation of infection depending on the inoculation procedure (see text).
+4, full spread; 0, no detectable spread. For details see ref. 35.

[b] The virulent and the avirulent strains were grown in three different germinal layers of CAM maintained in vitro and their glycoproteins were analysed by polyacrylamide gel electrophoresis. For details see ref. 35.

[c] not tested.

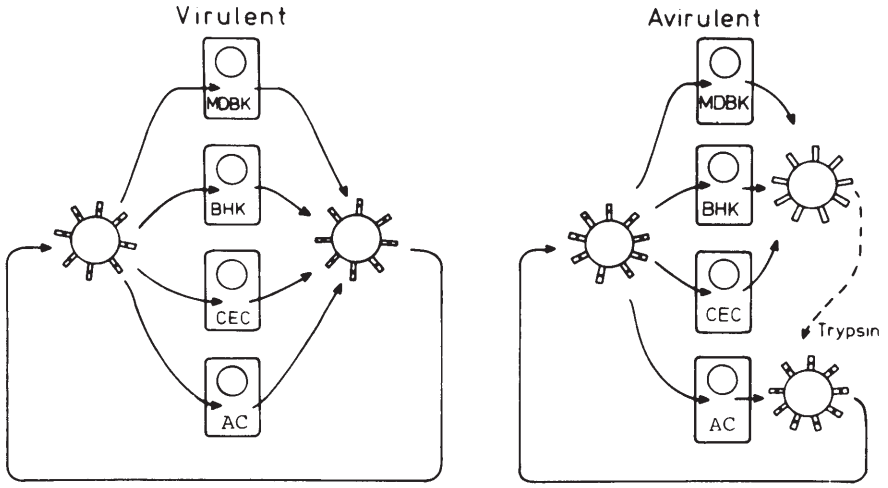

Fig. 4 Host range of virulent and avirulent strains of NDV as determined by the susceptibility of the envelope glycoproteins to proteolytic cleavage. Avirulent strains produce fully infective virus with cleaved glycoproteins in the allantoic cavity (AC) of chick embryo. In MDBK, BHK-21 and primary chick embryo (CE) cells, they produce noninfectious virus with uncleaved glycoproteins, which can be converted, however, into active virus by trypsin treatment. Virulent strains produce fully infectious virus with cleaved glycoproteins in all cells analyzed.

6. *Change in Cleavability of Glycoprotein F Causes Changes in Virulence* A comparison of the viral proteins by peptide mapping has demonstrated that the structures of internal proteins such as NP and L are highly conserved among virulent and avirulent strains, whereas the external glycoproteins show considerable structural variations.[37] In addition, the degree of the variations seems to correlate with the difference in virulence. It is, therefore, a reasonable assumption that virulent and avirulent strains might arise from each other by spontaneous mutations in glycoprotein genes leading to different sensitivity to proteolytic cleavage. In agreement with this concept, a chemically induced mutant could be isolated from an avirulent strain La Sota, which undergoes efficient cleavage of F_0 in tissue culture cells and shows, in parallel, increased virulence for chick embryo.[38] A comparative analysis by tryptic fingerprints between the wild type and the mutant indicated that changes had occurred only in F gene but not in those coding for the other major proteins (Fig 5). These results indicate that the difference in cleavability is determined by variations in genuine structural properties of the glycoprotein and provide a strong support for the importance of cleavability in the viral pathogenesis.

7. *Antigenic Analysis of Viral Glycoproteins* In spite of apparently adequate protection measures, *i.e.*, vaccination of birds with an appropriate avirulent strain such as B_1, Newcastle disease still recurs periodically. In light of the studies described above, new isolates can now be examined for their virulence *in vivo* and for cleavability of their glycoproteins *in vitro*.[29] However, due to high cross reactivity of antisera prepared against the virus in a conventional manner, there has been no sure way to distinguish the isolates antigenically. Other paramyxoviruses including human pathogens are in a similar situation, which hampers more detailed diagnostic and ecological analyses of paramyxoviruses. Thus, there is clearly a need for a greatly improved method for antigenic analysis of these viruses. We prepared monoclonal antibodies to one of the glycoproteins, HN, of NDV, defined antigenic domains in the glycoprotein, and used the antibodies for strain comparisons.[39]

An avirulent strain D_{26} isolated recently in Japan[40] was used as the antigen, and hybridomas secreting anti-HN antibodies were obtained. These hybridomas were classified into three groups, I, II and III, by inhibition tests for hemagglutination, neuraminidase and infectivity, by competitive binding assays and by frequency of the occurrence of antigenic

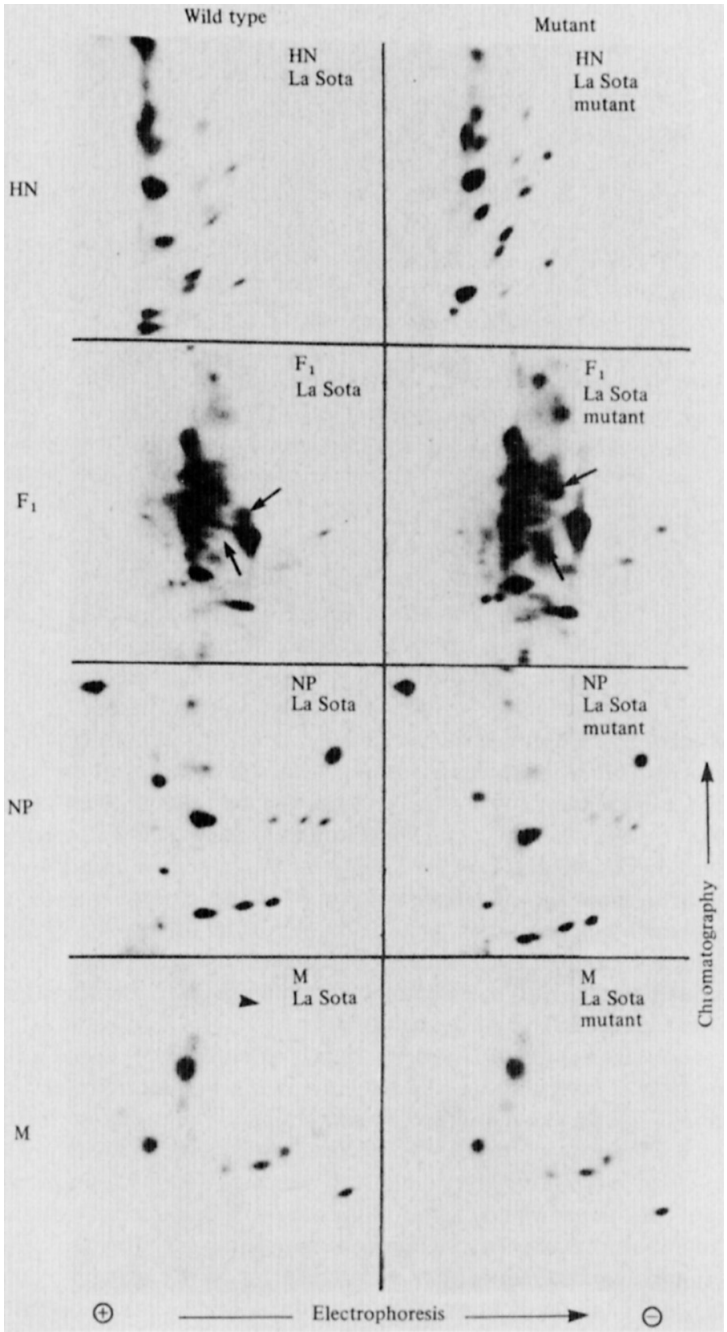

Fig. 5 Trypyic peptide patterns of the major structural proteins of wild type and mutant La Sota. For details see ref. 38).

variants in the presence of each antibody. Group I antibodies were found to recognize a region close to the biologically active (hemagglutination-neuraminidase) site and to exhibit high neutralizing activities. The Group II antibodies are to another region distinct from the group I domain and distant from the biologically active site and thus inhibit the activities only sterically. This site seemed to be less important than the group I region in raising neutralizing antibody. Group III antibodies are to a region overlapping group I determinant, but lack neutralizing activity.

The reactivity of the antibodies with a series of virulent and avirulent strains was examined. The results obtained for individual antibodies representing each of the three groups were presented by octagonal diagrams (Fig. 6). The group I antibody exhibited high reactivities in enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition (HI) and neutralization with all of the strains tested except for Herts strain in HI test. Thus, the diagram showed a relatively regular octagon. In contrast, the group II and III antibodies failed to bind to some strains as judged by ELISA. Further, even when binding occurred, there was no or only small inhibition of hemagglutination or infectivity in some cases. These results indicate that HN glycoprotein contains regions antigenically conserved as well as those subject to change, the former being recognized by group I antibodies and the latter by group II and III antibodies. However, the antigenic changes detected here seemed not to be correlated with virulence.

The conserved domain recognized by group I antibodies is important, as noted above, in raising neutralizing antibodies. This would explain the high cross reactivity among NDV strains by conventional serology and give a *rationale* for the protection of birds with an avirulent strain such as B_1. From the above results of antigenic variations, however, it is predicted that antibodies may sometimes be elicited only to the conserved region of an incoming virulent virus. In such a case, prophylaxis may not be fully effective since neutralization is far more extensive if group I and II antibodies are used in combination.[39] When two or more avirulent strains are used for vaccination and antibodies are elicited to plural domains, more effective immunoprophylaxis may be achieved.

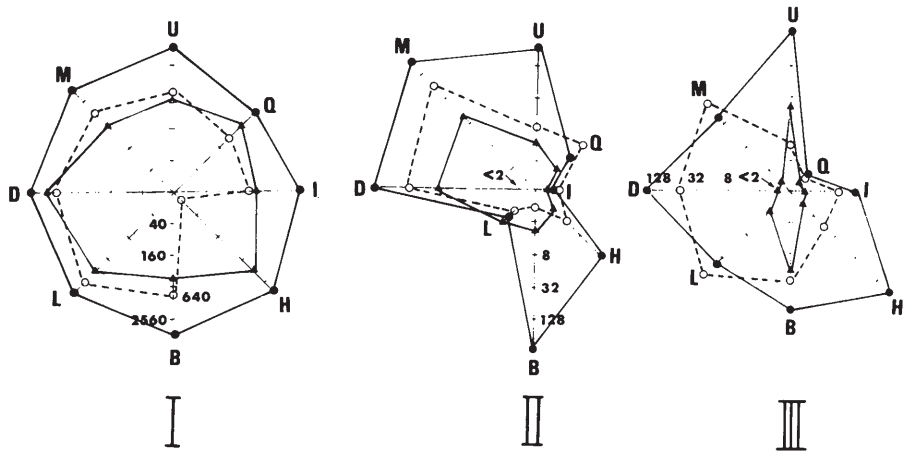

Fig. 6 Reactivity patterns of group I, II and III antibodies with heterologous virulent strains, Miyadera (M), Italien (I) and Herts (H), and avirulent B_1 (B), La Sota (L), Queensland (Q) and Ulster (U) strains. D, homologous strain D_{26} (avirulent). The titers for each strain of an antibody were plotted on the individual radial scales extending from the center toward the direction of each apex of an ocyagon. The points of the 8 strains were then lined with one another for the individual tests. ●——●, ELISA ($\times 10^{-3}$); ▲——▲, neutralization ($\times 10^{-2}$), O– – – –O, hemagglutination inhibition ($\times 10^{-2}$). For details see ref. (39).

MECHANISM OF PERSISTENT INFECTION WITH SENDAI VIRUS

1. *General Characteristics of Sendai Virus Carrier Culture* Quite distinct from acute infections are those in which the virus persists for months and years, *i.e.*, persistent infections. This type of infection is conceived to be associated with a variety of pathogenic processes and clinical manifestations.[1] With paramyxoviruses these involve subacute sclerosing panencephalitis caused by measles virus and multiple sclerosis for which parainfluenza type 1 and measles virus are the causative candidates. To understand this type of virus infection, it may be helpful to define the factors which are involved in the induction of persistent infections in cultured cells.

Maeno *et al.*[41] and Nagata *et al.*[42] established Sendai virus-persistent infections in HeLa and BHK (baby hamster kidney) cells, respectively. These carrier cultures were established by cultivating a small fraction of cells which survived the lethal infection with the virus, have been maintained in our laboratory for more than 10 years, and possess the following characteristics.[22, 42, 43, 44, 45] They exhibit virus-specific immunoflurescence in nearly 100% of the cells. They are highly resistant to the challenge of infection with prototype Sendai virus but are fully sensitive to heterologous viruses. Neither is interferon involved in the maintenance of the viral carrier state nor is antivirus serum able to cure the persistent infection. The carrier cultures are shedding only a small amount of virus particles at the temperature for maintenance of the culture (36° C), but significant levels of virus production are induced by temperature shift down to 32°C. The released virus at 32°C is temperature sensitive (ts) in the synthesis of HN and M proteins. From the last observation, ts mutants have been suggested to play some role in the maintenance of the persistent infection.

On the other hand, there is a series of evidence indicating the requirement of defective interfering virus particles for persistent infections with Sendai virus[46] as well as the other viruses.[47] The DI particles are found in very small amounts in usual preparations of various viruses, and can be obtained in large amounts when viruses are passaged repeatedly at unusually high multiplicities of inoculation. DI particles have the following properties in common: (1) they contain normal structural proteins; (2) they contain only a part of the virus genome; (3) they can reproduce only in cells infected with homologous standard virions which act as helpers; and (4) they specifically interfere with the multiplication of the homologous standard virus. If BHK cells are infected simultaneously with standard and DI Sendai viruses, they all survive and enter a state of persistent infection.[46] Both standard and DI-particle-size genomes persist in these cells.

2. *Establishment of Sendai Virus Persistent Infection by ts Mutants without Aid of DI Particles* From the observations described above at least two factors, DI particles and ts mutants, have been proposed to play roles in the establishment of Sendai virus persistent infection. However, the above studies made no parallel analysis of the two factors but documented or emphasized so far the relevance of only one or the other factor. We have therefore looked for the presence of DI particles in our BHK-Sendai virus carrier culture on one hand and, on the other, investigated whether the ts mutants from the carrier culture can establish a persistent infection in normal cells.[48]

When the carrier culture was analyzed for RNA species contained in intracellular nucleocapsids, several RNAs smaller than 50S were detected in addition to genome-size 50S RNA (Fig. 7a, b), indicating that the cells were producing both complete and DI-size RNAs. This supported the relevance of DI particles to the establishment of persistent infection. Then the incubation temperature of the carrier culture was shifted from 36° to 32° C and ts mutants were isolated. The ts virions were purified by centrifugation through potassium tartrate gradients so that they comprised only standard virions containing 50S RNA genome (Fig.

7d). These virions caused little cytopathic changes in newly prepared normal cells, and the infected cells were readily cultivated by serial passages. Nearly 100% of the cells exhibited virus-specific immunofluorescence and were resistant to infection with wild type Sendai virus. Thus, the ts virions alone could indeed establish a persistent infection. RNA species in the nucleocapsids accumulated in these cells were examined, and it was found that only 50S RNA was present (Fit. 7f, g). This indicates that no DI RNAs were generated during maintenance of the newly established persistent infection with the ts virus. Therefore, DI particles are required neither for initiation nor for maintenance of the persistent infection. Furthermore, the ts virus interfered strongly with the replication of wild type virus, and

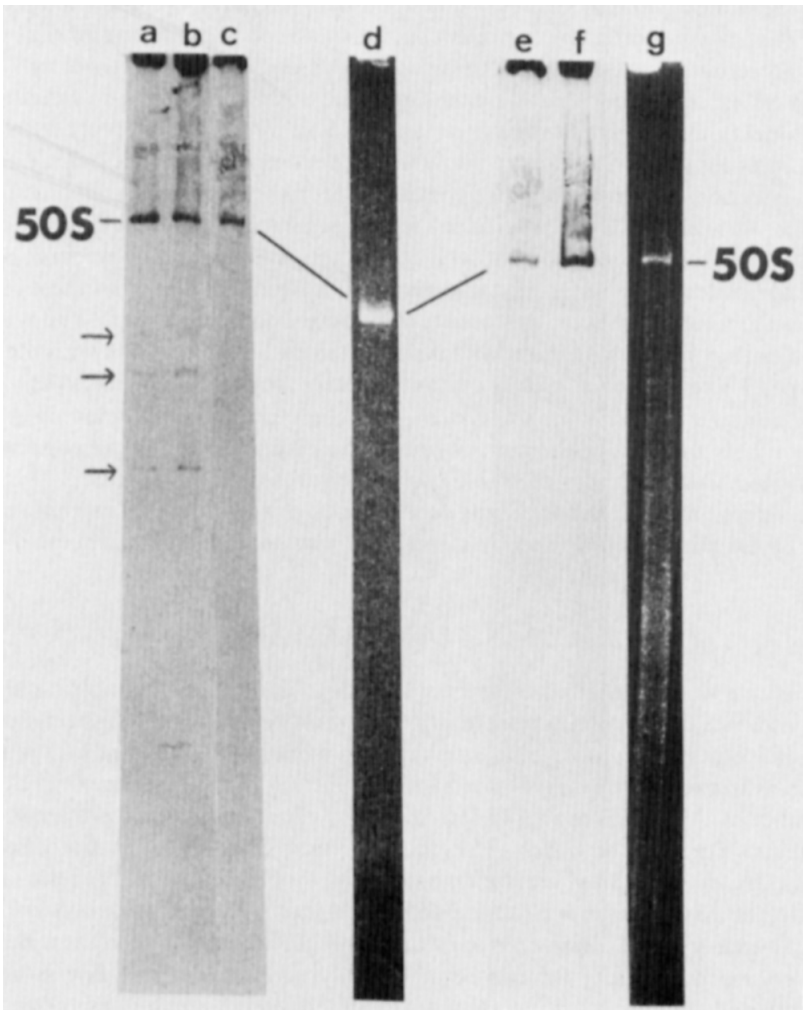

Fig. 7. Polyacrylamide gel electrophoresis of RNAs isolated from nucleocapsids in BHK-Sendai virus carrier cultures of passage 477 (a) and 488 (b), from virions of a ts mutant (d), and from nucleocapsids in cells of 2nd (f) and 7 th (g) passage following infection with the ts mutant. Nucleocapsids in lytically infected cells with wild type virus are shown to contain only 50S RNA (c, e). Arrows indicate RNAs smaller than genome size 50S RNA. The RNAs on gels a, b, c, e and f had been labeled with ^{3}H-uridine and were detected by fluorography, whereas those on gels (d) and (g) were detected by staining with ethidium bromide. For details see ref. (48).

infection with a mixture of ts and wild type allowed the cells to survive and also resulted in a persistent infection.[48]

3. *Two-Step Mechanism for the Establishment of Sendai Virus Persistent Infection* Our results described above indicate that both DI particles and ts mutants are involved in the maintenance of Sendai virus persistent infection. The question that now arises is which factor is essential or are both required. Based upon the following considerations, we suggest that the establishment of the persistent infection may involve two crucial steps which would be mediated by each of the factors.

Routinely, the persistent infection is initiated by cultivating a small fraction of cells which survived the initial lethal infection with wild type virus. Because of the widespread occurrence of DI particles among animal viruses,[49] the stock virus may always contain a trace level of DI particles. This may be sufficient to protect such a small cell population and thus to initiate a persistent infection in these cells. During serial passages, however, these cells encounter occasional crises, indicating that DI particles alone are not sufficient to maintain a stable persistent infection. DI particles require standard wild type virus to reproduce themselves and, thus, in order to persist, have to allow wild type to grow to some extent. Therefore, the persistent infections initiated by DI particles are probably maintained by a subtle balance between the intracellular levels of virulent wild type and DI particles. Once such a balance declines toward the predominance of wild type, the system probably undergoes crisis.

The carrier culture then enters a stable phase no longer showing crisis. Just concurrently with, but never antecedently to, this phase, ts mutants begin to evolve.[50] The ts mutants are poorly cytopathic, multiply without wild type and, in addition, interfere with the replication of wild type. Therefore, once such ts mutants evolve, they tend to replace the virulent wild type and ultimately expel it from the system, thus eliminating crisis encounter in the culture. Consequently, ts mutants with poor cytopathogenicity and interfering capacity could be readily isolated from a variety of stable carrier cultures of Sendai virus.[51, 52] In summary, persistent infection with Sendai virus is establised by a two-step mechanism, namely, initiation by DI particles followed by stabilization by endogenously generated ts mutants.

CONCLUDING REMARKS

In this article we summarized our recent studies to define the mechanism underlying the processes of acute and persistent infections with paramyxoviruses. It has been demonstrated that a relatively simple molecular mechanism, namely, the susceptibility of the viral glycoproteins to proteolytic cleavage, is highly important for the pathogenesis by NDV. The susceptiblility to cleavage is probably determined by genuine structural properties of the viral glycoproteins. These studies may represent the first case in which the disease-causing mechanism by a virus has been elucidated by biochemical and genetic approaches. Apparently, the investigation was facilitated by the use of NDV as a model system, since NDV comprises a variety of well-characterized strains and grows well in appropriate hosts so that it can be prepared in quantity for biochemical analysis. However, the fine structure of the cleavage site and the host protease (s) required for cleavage remain to be elucidated.

Following our studies, avian influenza virus has been found to be quite similar to NDV; its surface glycoprotein HA acquires biological activities through proteolytic cleavage[53, 54] and this activation is also of great importance for the viral pathogenicity.[55] In addition, specific differences in amino acid sequence at the cleavage site have been determined which can explain the variations in cleavability between virulent and avirulent strains.[56, 57, 58] Another good evidence indicating the importance of viral surface proteins for pathogenesis has been

presented with murine reovirus.[59] Type 3 reovirus causes a necrotizing encephalitis (without ependymal damage) which is always fatal, whereas type 1 causes ependymal cell damage (without neuronal necrosis) and hydrocephalus, which is nonlethal. Using gene reassortant clones derived from crosses between type 1 and 3, it has been demonstrated that only one gene, S1 segment, of 10 segmented reovirus genomes, is responsible for the differences in cell tropism. Probably, the differences are due to the specific interaction of $\sigma 1$ outer capsid protein (coded for by the S1 segment) with receptors on the surface of either the ependymal or neuronal cells.

Since the discovey of fixed rabies virus by Pasteur, adaptation of a virus to different or unnatural hosts is known to result in attenuation of the virus and has been a major strategy for the development of live vaccines. Thus, there is also a way to study viral pathogenesis by comparing wild and attenuated strains. Several works have recently been done along this line. Following are the examples on mumps virus and poliovirus. With mumps virus, attenuation is achieved by adaptation of human isolates to chick embryo. The attenuated mumps virus is fully replicative in chick embryo cells but not in mammalian cells where original wild strains grow well.[60, 61] Analysis of infection with attenuated strains in an established line (Vero) of African green monkey kidney cells has suggested that attachment, penetration and early genome expression take place normally, whereas the subsequent expression at a late stage and amplification of the viral genome may be strongly restricted.[62] In addition, this restriction has been suggested to be modulated at least in part by some host cell factor (s) whose synthesis is sensitive to actinomycin D, an inhibitor of DNA dependent RNA synthesis (manuscript in preparation).

The primary structures of the genome of wild type poliovirus (Mahoney) and Sabin I vaccine strain were determined[63, 64, 65] and compared with each other.[65] The results indicated that the vaccine strain underwent a total of 57 point mutations in a whole single-stranded RNA genome of 7441 bases and that 21 of them resulted in amino acid substitutions. Further, the amino acid substitutions were found to be accumulated near the N-termini of capsid protein VP1. Thus, the change in VP1 is probably responsible for attenuation of poliovirus. One of the most important and serious problems in prophylaxis with live vaccines is the occurrence of a wild type revertant; therefore, the basis for genetic stability should be elucidated with any live vaccines. In this respect, the above study of poliovirus makes quite an important beginning.

Persistent infections are known to arise almost commonly in cells infected with a variety of viruses. The proportion of infected cells, the mode of expression of viral genome, and the amount of virus produced by the cells vary greatly depending on the virus as well as the cell type used. An interesting feature they share may be their presumed involvement in a variety of chronic diseases of unknown etiology. In addition, persistent infections are of biological and genetic interest, since they generate deletion mutants (DI particles) and new virus polulations such as ts mutants. However, we have not attempted here to review the whole picture of persistent infection but have confined our remarks to Sendai virus persistent infection in cell culture. The major point drawn concerns the specific roles of DI particles and ts mutants in the establishment of the persistent infection. The former is essential to its initiation and the latter to its stabilization. Previous studies have emphasized the importance of either of the two factors and have often neglected the other; therefore, the conclusions have often conflicted with each other. Our two-step theory would overcome this confused situation.

Molecular biology applied toward understanding viral diseases is just coming of age, as we illustrated by several examples in which questions of viral pathogenesis can be answered by biochemical analysis of animal viruses and their infected cells. A large body of works focused on host reactions to virus infection has also accumulated in the last decade and further studies

along this line are now in progress. These continued efforts from various aspects will provide valuable information and concepts useful for the more effective control of virus diseases.

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